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# Distribution of Primary Lung Carcinomas in Relation to Time as Determined by Histochemical Techniques[1,2]

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SUMMARY

**The 1,037 neoplasms with an original anatomic diagnosis of primary pulmonary carcinoma that were observed at necropsy at the Los Angeles County Hospital from 1927–57 were reviewed. Of these, 956 met the criteria for inclusion in this study. The neoplasms were reclassified according to two criteria: 1) a modification of Liebow's method in which hematoxylin-and-eosin-stained sections were used; 2) that of the World Health Organization in which, in addition, sections specially stained to demonstrate mucin and keratin were used. In our series approximately 30 percent was poorly differentiated or undifferentiated and close to 10 percent was the uncommon giant and/or spindle-cell variant. Most of these undifferentiated and poorly differentiated neoplasms contained acid mucopolysaccharide and were classified as adenocarcinoma. The percentage distribution of the various histologic types, especially the adenocarcinoma group, differed significantly according to the criteria used. Our data agree with other published reports which show that the frequency of lung carcinoma is increasing disproportionately to all necropsies. However, the roles played by various histologic types differ among studies. Independent of criteria used in our series, the proportion of epidermoid carcinomas in men is not increasing, while the proportion of adenocarcinomas apparently is increasing. Our ratios of epidermoid and undifferentiated carcinomas to adenocarcinomas are decreasing, while ratios reported by Kreyberg and others are increasing. Although this contrast is due partially to difference in interpretations by pathologists as well as to variations in methods and criteria, the Los Angeles County Hospital material is undeniably different from that reported elsewhere. The reasons for these differences need explanation, and further studies of both clinical and necropsy material from the Los Angeles area are indicated.—J. Nat. Cancer Inst. 27: 1227–1271, 1961.**

RECENT STUDIES in Norway (*1–5*), England (*6*, *7*), and the United States (*8–12*) demonstrated a marked increase in the incidence of lung cancer in males during the past 40 years. Numerous authors (*1–5*, *7*, *13*),

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on the basis of histologic studies of necropsy and surgical material, concluded that the increase is manifested almost exclusively in the epidermoid and undifferentiated types of neoplasms. The frequency of adenocarcinoma has remained relatively constant. Several investigators (*3–5, 11, 13–15*) suggested that exogenous factors, especially cigarette smoking, are of greater importance in the production of epidermoid and undifferentiated carcinomas than of adenocarcinomas. While universal agreement in relation to this is lacking (*16, 17*), by far the predominant opinion supports this concept.

Since the necropsy series of the Los Angeles County Hospital was studied previously (*8, 9*), was very large, and covered a long period of time, this source material offered an excellent opportunity to investigate differential trends of lung cancer by histologic type. Therefore, a study was undertaken to classify histologically all lung carcinomas observed in the necropsies of this hospital for the 31 years, 1927–57. Special emphasis was directed to determine whether the changing distribution of histologic types observed elsewhere was manifested in our material. Because of limitations in the histories regarding environmental exposure and other factors presumably related to pulmonary carcinogenesis, any etiologic implications of our morphologic findings were necessarily limited to the postulated relationships between histologic types and exogenous factors.

Two different sets of criteria were used for the designation of the lung tumors: One, in general use in this country, was based on the system of Liebow (*18*); the other copied the World Health Organization method devised by the Expert Committee, which included both Liebow and Kreyberg (*19, 20*). This dual classification provided an opportunity to analyze and compare the data resulting from the application of each.

## MATERIALS AND METHODS

A total of 55,329 necropsies was performed at the Los Angeles County Hospital from 1927–57. This necropsy series represents approximately 35 percent of hospital deaths and includes all age groups and both sexes. Because of the unique nature of the indigenous residents of this area, the population was arbitrarily divided into Caucasian, Negro, Mexican, Oriental, and Indian. For this study, Caucasian refers to all individuals who were not included in any other racial or ethnic group. In some ethnic groups, the numbers of neoplasms were too small to determine time trends. A portion of this same necropsy series was reviewed by Steiner (*9*) in his extensive study of epidemiologic characteristics of cancer by site for the years 1918–47. While the cases he reviewed began in 1918, the unavailability of tissues or slides for the 11 lung carcinomas observed at necropsy between 1918–28 forced us to begin our study with the records of the year 1927.

All necropsies in which the original anatomic diagnosis of lung cancer was shown as the cause of death, and all those in which lung cancer was listed as an incidental finding, were studied in detail. In a total of 1,037

necropsies performed during this period, an original anatomic diagnosis of lung cancer was made. Eighty-one were eliminated. These included metastatic carcinoma, 46; primary and secondary sarcoma, 8; lymphoma, 12; and inflammatory or indefinite conditions, 15. Of the remaining 956 considered to be primary lung carcinoma, microscopic sections in 54 were either unavailable, technically poor, or revealed equivocal neoplasms by the WHO criteria.

The "blind technique" was used to review all histologic material. Possible bias incidental to the knowledge of year or autopsy numbers that were assigned consecutively was thus obviated. Finally, the study material for review was randomly selected. Inasmuch as the criteria for histologic classification have changed and unanimity does not exist in the classification of lung cancers, all tumors included were reclassified by one of us (D.L.H.). Any theoretical bias resulting from variation in interpretations was checked by a study of internal consistency. The change in technique from frozen to paraffin sectioning in 1945 was reflected in the improved quality of the slides. This made it necessary to divide the neoplasms into two groups—those diagnosed at necropsy between 1927–44 and those between 1945–57. Separate but comparable analyses were performed on each group, with the use of the two sets of criteria mentioned previously.

*Histologic criteria.*—The criteria used for the designation of lung tumors were: 1) one of the methods in use in the United States, based on the system of Liebow (*18*), and 2) the method since adopted by the WHO, similar to the classification used by Kreyberg in 1952 (*19*, *20*).

Modifications were made in the Liebow criteria, in that no special stains were used for the demonstration of mucin and in the method of classifying the epidermoid and anaplastic carcinoma group. Using Liebow's scheme, we erroneously designated as epidermoid carcinomas, tumors composed of cytocohesive clear cells growing in nests and giant-cell carcinomas with cells having abundant eosinophilic cytoplasm. So as to categorize adequately the heterogeneous anaplastic tumors in our series, we arbitrarily divided these neoplasms into three subgroups: 1) small- or oat-cell, 2) large-cell, and 3) giant and spindle-cell carcinomas that were not designated as epidermoid carcinomas. In contrast, the WHO system was used with virtually no change. Our modification of the criteria published by Liebow will be referred to as A criteria and the unaltered WHO system as the B criteria. In classifying tumors according to the A criteria, we stained sections only with hematoxylin and eosin and made designations on the basis of morphology. In addition to the hematoxylin-and-eosin-stained slides, sections used for classification on the basis of B criteria were specially stained for demonstration of keratin and mucin for poorly differentiated and undifferentiated neoplasms; thus, these tumors were categorized by morphology and histochemical reactions. However, the differentiated neoplasms and small-cell anaplastic carcinomas (fig. 23) were classified from hematoxylin-and-eosin-stained sections for both A and B criteria.

Initially, we planned to use Liebow's scheme or A criteria exclusively on the assumption that it was comparable to the WHO system or B criteria. The need for the delineation of distinct criteria for A and B became manifest after the review by Dr. Kreyberg of 100 randomly selected tumors from our series. Striking differences were observed in several categories when our diagnoses according to the A criteria were contrasted with Kreyberg's (*21*) interpretations with the B criteria. Most of the carcinomas that were diagnosed as 1) giant- and spindle-cell anaplastic (figs. 1, 18, 20, and 22); 2) giant-cell epidermoid (figs. 2, 4, and 19); 3) mixed epidermoid and adenocarcinoma (figs. 5, 15, and 16); 4) epidermoid composed of cytocohesive cells (figs. 3, 9, 10, 11, and 12); and 5) large-cell anaplastic (figs. 4 and 17) according to our A classification were interpreted as anaplastic adenocarcinoma by Kreyberg. Kreyberg's interpretations were confirmed by us after our use of histochemical reactions. These included periodic acid-Schiff reaction (*22*), Alcian blue-saffron (*23*), Alcian blue-orange G (*24, 25*), and Mayer's mucicarmine (*26*). The periodic acid-Schiff reaction, Alcian blue, and Mayer's mucicarmine demonstrate mucopolysaccharide, while phloxine B or erythrosin with saffron (*23*) or orange G (*25*) stain keratin. With the use of these stains intracytoplasmic acid mucopolysaccharide was consistently demonstrated in these tumors. The stains we used to demonstrate acid mucopolysaccharide were superior to the ones used for the detection of keratin. While sections stained with hematoxylin and eosin were sufficient for the designation of differentiated tumors, routine staining was inadequate for the poorly differentiated and undifferentiated tumors listed. Because of these differences, we classified our material further by the WHO or B criteria.

Histologic sections from 118 poorly differentiated and undifferentiated tumors prepared from frozen sections were destained and restained with Alcian blue-saffron. For the sections prepared from paraffin blocks, the first three stains previously listed were used for all 204 poorly differentiated and undifferentiated tumors. The use of Mayer's mucicarmine was abandoned after sections of 81 neoplasms were stained because frequently the findings did not conform with those from the other three stains.

The following criteria are presented (phrases in italics denote our modifications):

| A | B |
|---|---|
| Epithelial | Epithelial |
| Adenocarcinomas—tumors showing obvious gland and acinar formation or containing intracytoplasmic mucus | Adenocarcinomas—acinar or papillary with or without mucus formation and tumors with a predominance of "large cells", some of which show gland formation and/or mucus production. (Our cytocohesive variant and giant- and spindle-cell adenocarcinoma conform to large-cell adenocarcinomas) |

| A | B |
|---|---|
| Epithelial—Continued | Epithelial—Continued |
| Bronchiolar—papillary adenocarcinomas neither metastatic to the lung nor originating in a bronchus, but apparently arising from the terminal bronchioles and/or alveoli | Bronchiolar—alveolar cell carcinomas—same as "bronchiolar" under A |
| Epidermoid—tumors showing keratinization, pearl formation, and the presence of intercellular bridges; *those composed of large polygonal cells growing in cohesive nests (referred to as cytocohesive tumors); giant- and spindle-cell tumors having abundant eosinophilic cytoplasm* | Epidermoid—highly, moderately, and slightly differentiated tumors showing varying degrees of keratinization and intercellular bridges |
| Anaplastic—small-cell anaplastic tumors, undifferentiated large-cell tumors, and *giant- and spindle-cell carcinomas not included in the epidermoid group* | Small-cell anaplastic—tumors composed of oval cells (oat cells) and/or small polygonal cells |
| | Large-cell undifferentiated—primary anaplastic large-cell tumors showing no differentiation toward epidermoid or adenocarcinomas |
| Mixed—tumors showing both adenomatous and squamous or *cytocohesive components* | Combined epidermoid and adenocarcinomas—tumors with features of both adenocarcinoma and epidermoid |
| Bronchial adenomas—tumors of the carcinoid, cylindroma, and muco-epidermoid types, both benign and malignant | Carcinoid tumors—having solid, trabecular or alveolar patterns |
| | Tumors of mucous glands—cylindromas; muco-epidermoid tumors. These are often polypoid tumors of the large bronchi and composed of cells arranged in acini or large rounded masses. The cells are variously mucus-producing, squamous or large polygonal, and undifferentiated. Frequently the mucus-producing cells differ little from those of the normal gland, but there may be considerable anaplasia |
| | Papillomas of the surface epithelium—epidermoid; epidermoid with goblet cells |
| Miscellaneous—sarcomas, unclassified tumors and inflammatory processes originally confused with neoplasm (only tumors apparently originating in the lung were included in the study) | Sarcomas—tumors similar to those arising in soft tissues generally |
| | Combined tumors of epithelial and mesenchymal cells |
| | Mesotheliomas of the pleura |
| | Tumors unclassified |
| Metastatic—tumors metastatic to the lung and lymphomas were excluded from the study | Metastatic—same as A |

*Review.*—All data were recorded on printed marginal punch cards as shown in figure 21. The four review procedures are described and are summarized in table 1; the reasons for the reviews are given in table 2.

A Criteria Methods

1) Sample review—Since the number of sections available varied with each neoplasm, a maximum of three sections from each was selected to determine the extent to which such a sample accurately reflected the variation in pattern seen in each tumor. In instances where more than three sections were available, those chosen for study were randomly selected. All available sections were used for those neoplasms having three or less. Occasionally, none of the lung sections in the sample review included the neoplasm.

2) First full review—All available slides and the pertinent information, recorded on the card after the sample review, were used. Data recorded included year of death, age, sex, race, locus of the tumor, metastatic sites, clinical diagnosis, primary anatomic diagnosis (made at the time of death), and the original histologic diagnosis.

TABLE 1.—Criteria and method of review in relation to time period

| Year | A criteria | B criteria |
|---|---|---|
| 1927–44<br>Frozen sections | Sample review<br>First full review<br>Consultants' review | Second full review |
| 1945–57<br>Paraffin sections | Sample review<br>Consultants' review on poorly differentiated and undifferentiated tumors | Second full review |

TABLE 2.—Comparisons of review methods with indications

| Time period | Comparison of review methods* | | Indications |
|---|---|---|---|
| 1927–44<br>Frozen sections | 1) First full review (A) | Sample review (A) | To check reliability of sample review |
| | 2) First full review (A) | Consultants' review (A) | To check conformity of the one pathologist (D.L.H.) who reviewed all cases |
| | 3) First full review (A) | Second full review (B) | To measure effect of changing criteria |
| 1945–57<br>Paraffin sections | 4) Sample review (A) | Second full review (B) | Combined with comparison 3 above to measure effect of changing criteria |

*Criteria used shown in parentheses.

3) Consultants' review—Five additional pathologists were consulted for this phase of the study. Each used the identical information and histologic sections as noted under 2).[6] The consultants' review was significantly facilitated by the invaluable contributions of Dr. Arthur Purdy Stout (*27*), Dr. Averill Liebow (*28*), Dr. Lauren

[6] We wish to express our gratitude to the following members of the Pathology Department of the Los Angeles County Hospital for their assistance in this study: Dr. E. M. Butt, Chief Pathologist; Dr. W. K. Bullock, Surgical Pathologist; Dr. A. E. Hirst, Associate Professor of Pathology, College of Medical Evangelists; Dr. H. A. Edmondson, Professor of Pathology, University of Southern California School of Medicine; and Dr. Dorothy Tatter, Head, Autopsy Pathologist, Los Angeles County Hospital.

Ackerman (*29*), and members of the Tumor Tissue Registry, Los Angeles, California (*30*), who also classified some of the poorly differentiated and undifferentiated tumors.

B Criteria Methods

4) Second full review—This review was identical to the first full review described previously except that the B criteria were used.

Originally only the first three reviews were planned for each neoplasm by the use of A criteria. Studies were to be done on frozen and then on paraffin material. All three reviews were completed on the former. However, during the sample review of the paraffin-sectioned tumors, the necessity for a change in criteria became evident. Therefore, a second full review with the use of B criteria was done on all tumors. Consequently, for the neoplasms classified exclusively from frozen tissue, all four reviews were completed, while for the neoplasms prepared from paraffin-sectioned tissue, only the sample review and the second full review were undertaken. Because of the consistent agreement between the reviewing pathologist's and consultant's diagnoses on the frozen-sectioned neoplasms, a consultants' review was sought only for the poorly differentiated and undifferentiated tumors prepared from paraffin sections.

## RESULTS

The results will be presented under four categories:

1) *General*—brief background information of the necropsies in the study.

2) *Internal consistency (of neoplasms histologically diagnosed from frozen sections)*—a comparison of the diagnoses of the full review with those of the sample review and the consultants' review.

3) *Criteria differences*—a detailed comparison of the two sets of criteria and their effects on distribution of histologic types.

4) *Demographic and clinical characteristics of the cases*—a presentation of results for each criteria by year of death, sex, age, and ethnic group, and a presentation of data relating locus to histology.

### General

The 1,037 necropsies with an original anatomic diagnosis of primary lung carcinoma were reviewed and 956 met the criteria for inclusion in this study. The number of carcinomas by time periods and the criteria used for diagnosis are given in table 3. The proportion of neoplasms diagnosed from frozen tissues for which a definite histologic designation could not be made was nearly constant for all time periods, and the use of A or B criteria did not alter this finding. Approximately 40 neoplasms prepared from frozen tissues that were considered primary lung carcinoma could not be reclassified for this study because the original microscopic slides were unavailable.

Over 60 percent of the neoplasms were studied from paraffin sections and slides were available for all but two neoplasms. We found marked

TABLE 3.—Distribution of lung carcinomas observed at necropsy by criteria and time

| | Frozen sections | | | | Paraffin sections | | | | Total |
|---|---|---|---|---|---|---|---|---|---|
| | 1927–34 Number | 1935–39 Number | 1940–44 Number | 1927–44 Number | 1945–49 Number | 1950–54 Number | 1955–57 Number | 1945–57 Number | 1927–57 Number |
| Necropsies considered to be primary lung carcinomas, total | 53(100. 0)* | 111(100. 0) | 192(100. 0) | 356(100. 0) | 184(100. 0) | 229(100. 0) | 187(100. 0) | 600(100. 0) | 956(100. 0) |
| A criteria | | | | | | | | | |
| Lung carcinomas reclassified by D.L.H. | 46(86. 8) | 98(88. 3) | 168(87. 5) | 312(87. 6) | 174(94. 6) | 213(93. 0) | 172(92. 0) | 559(93. 2) | 871(91. 1) |
| Lung carcinomas, but not reclassified by D.L.H.† | 7(13. 2) | 13(11. 7) | 24(12. 5) | 44(12. 4) | 10(5. 4) | 16(7. 0) | 15(8. 0) | 41‡(6. 8) | 85‡(8. 9) |
| B criteria | | | | | | | | | |
| Lung carcinomas reclassified by D.L.H. | 47(88. 7) | 96(86. 5) | 167(87. 0) | 310(87. 1) | 180(97. 8) | 227(99. 1) | 185(98. 9) | 592(98. 7) | 902(94. 4) |
| Lung carcinomas, but not reclassified by D.L.H.† | 6(11. 3) | 15(13. 5) | 25(13. 0) | 46(12. 9) | 4(2. 2) | 2(0. 9) | 2(1. 1) | 8(1. 3) | 54(5. 6) |

* Percentages in parentheses.

† Neoplasms definitely considered to be primary lung carcinoma, but microscopic slides were unavailable, technically poor, or slides did not contain primary neoplasm.

‡ Neoplasms (33) not reclassified histologically by A criteria because the three slides included in the sample review did not contain the primary pulmonary neoplasm.

differences between the histologic diagnoses in the sample review with the use of A criteria and in the second full review with the use of B criteria. These differences primarily reflected the variation in the number of sections available for reviews rather than differences resulting from the application of A and B criteria. For 33 neoplasms prepared from paraffin blocks, a histologic diagnosis was not possible by A criteria because the three slides of lung included in the sample review did not contain a section of primary tumor. However, the routine use of all sections in the full review for the same 33 neoplasms demonstrated the presence of primary tumor, which made a histologic diagnosis possible by B criteria.

## Internal Consistency

Despite the recognized diagnostic limitations of the frozen-section material, comparisons were made between each of the three reviews to check the validity of the sample slides and the consistency and compatibility of the reviewing pathologist's and consultant pathologist's diagnoses. Unfortunately, the paraffin-section material could not be used for a study on internal consistency since only a sample review and second full review were made on these. The results of comparing the first full review with the remaining reviews for neoplasms prepared from frozen tissues are shown in table 4. The agreement between the diagnoses of the first full review and the sample review was approximately

TABLE 4.—Comparison of first full review—A criteria—with other reviews, showing consistency of histologic diagnoses* †

| | Percent necropsies with first full review in | | |
|---|---|---|---|
| | Agreement | Disagreement | Number |
| (A criteria) | | | |
| Sample review | 88.1 | 11.9 | 285‡ |
| 1 section with tumor | 95.0 | 5.0 | 100 |
| 2 sections with tumor | 82.8 | 17.2 | 128 |
| 3 sections with tumor | 87.7 | 12.3 | 57 |
| (A criteria) | | | |
| Consultants' review | 86.9 | 13.1 | 312 |
| Original histologic diagnosis | 61.5 | 38.5 | 252§ |
| (B criteria) | | | |
| Second full review | 77.2 | 22.8 | 312 |

*Frozen section material.
†Excludes necropsies in which 1) lung cancer was not evident from the sections.
‡Excludes necropsies in which 2) no section of lung tumor was available for the sample review.
§Excludes necropsies in which 3) original diagnosis was indefinite or not stated.

90 percent, with some variations being noted in relation to number of sample sections available. Differences in diagnoses occasionally resulted when additional sections of lung or metastases added to the full review revealed variations in histologic patterns, particularly when a progression from differentiated to anaplastic tumor existed. The pathologist and the consultants agreed on 87 percent of the diagnoses. The original diagnoses and those following the first full review showed little agreement. In approximately 30 percent of the patients necropsied, the original histologic diagnosis was either indefinite or not given, and of those neoplasms that had been diagnosed histologically, there was 62 percent agreement with the first full review diagnosis.

### Diagnoses as Affected by Differences in Criteria

Differences in the diagnoses resulting from the use of the A and the B criteria were observed with carcinomas diagnosed from frozen-section preparations when the first full review was compared with the second full review, and with carcinomas diagnosed from paraffin-section preparations when the sample review was compared with the second full review. Contrasting diagnoses were noted in 23 percent of the cases, as indicated in table 5. This substantial difference resulting from the use of A and B criteria is emphasized. However, this is still less than the difference observed when the first full review is compared with the original histologic diagnosis. The classification of the large-cell ana-

TABLE 5.—Classification of lung carcinomas by A and B criteria to show variations between histologic diagnoses

| | A criteria | | | | | | B criteria | |
|---|---|---|---|---|---|---|---|---|
| B criteria | Adenocarcinoma | Epidermoid | Anaplastic | Mixed* | Bronchiolar | Other† | Total | Percent |
| Adenocarcinoma | 95 | 36 | 45 | 37 | 10 | 54 | 277 | 29.0 |
| Differentiated | 80 | 1 | 7 | | 4 | 28 | 120 | 12.6 |
| Cytocohesive | 2 | 24 | | 34 | 6 | 1 | 67 | 7.0 |
| Giant and spindle cells | 13 | 11 | 38 | 3 | | 25 | 90 | 9.4 |
| Epidermoid | | 256 | 6 | | | 20 | 282 | 29.5 |
| Anaplastic | | 7 | 226 | | | 18 | 251 | 26.3 |
| Small | | 6 | 222 | | | 11 | 239 | 25.0 |
| Large | | 1 | 4 | | | 7 | 12 | 1.3 |
| Mixed* | 1 | 11 | | 21 | | 2 | 35 | 3.7 |
| Bronchiolar | 2 | | | | 25 | 3 | 30 | 3.1 |
| Other† | 3 | 3 | 5 | 1 | | 69 | 81 | 8.5 |
| Total A criteria | 101 | 313 | 282 | 59 | 35 | 166 | 956 | 100.1 |
| Percent | 10.6 | 32.7 | 29.5 | 6.2 | 3.7 | 17.4 | 100.1 | |

*Combined epidermoid and adenocarcinoma.
† Includes all other primary lung carcinomas reviewed for this study.

plastic carcinomas (poorly differentiated and undifferentiated carcinomas), which constituted the major source of differences in diagnoses, is presented in table 6. The morphologic and histochemical characteristics for the poorly differentiated and undifferentiated carcinomas are detailed in these two tables.

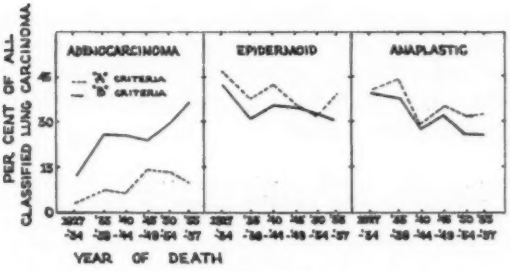
TABLE 6.—Comparison of A and B criteria classifications by morphologic and histochemical characteristics for undifferentiated and poorly differentiated lung carcinomas

| | | Morphology | Classification using A criteria | Classification using B criteria | Histochemical characteristics* | |
|---|---|---|---|---|---|---|
| | | | | | Acid mucopolysaccharide | Keratin |
| I | A | Large, clear, or eosinophilic cells growing in cohesive nests, some with pseudoglands | Epidermoid | Adenocarcinoma | + | − |
| | B | Same as above, but with keratinization or intercellular bridges | Epidermoid | Mixed | + | + |
| II | | Large, clear, or eosinophilic cells growing in cohesive nests with areas of obvious glands | Mixed | Adenocarcinoma | + | − |
| III | | Uncertain—tumors composed of large, clear cells or giant- and spindle-cell carcinomas, some with pseudoglands | Unclassified | Adenocarcinoma | + | − |
| | | | | Epidermoid | − | + |
| | | | | Anaplastic small cell | − | − |
| | | | | Anaplastic large cell | − | − |
| | | | | Other | − | − |
| | | | | Mixed | + | + |
| | | | Anaplastic | Adenocarcinoma | + | − |
| IV | | Same as III, but the giant cells have abundant eosinophilic cytoplasm | Epidermoid | Adenocarcinoma | + | − |
| | | | Mixed | Adenocarcinoma | + | − |

* + = Demonstrated; − = not demonstrated.

## Demographic and Clinical Characteristics of Cases in Histologic Diagnoses

*Time trends.*—The percentage distributions by histologic diagnoses as determined by each set of criteria are given in tables 7, 8, 9, and 10. Data for men are analyzed by the individual sequential time intervals, while for women the two inclusive time periods 1927–44 and 1945–57 are used. The proportions of epidermoid and anaplastic carcinomas in men are slightly lower when the B criteria were used. This is accompanied by a parallel increased proportion of adenocarcinomas. However, the trends as shown in text-figure 1 are relatively constant: A slight increase in adenocarcinomas is accompanied by a slight decrease in epidermoid and anaplastic carcinomas. The increase in adenocarcinomas and the accompanying decrease in the other two major groups, though not marked,




TEXT-FIGURE 1.—Comparison of percentage distribution for men of the three major histologic types of lung carcinomas as diagnosed by A and B criteria in relation to time.

assume significance when contrasted with virtually all published data (*1–4, 10, 13, 31*).

This apparent contradiction requires a detailed analysis. In men, adenocarcinomas comprise approximately 10 percent of all lung tumors when A criteria are used. Epidermoid and anaplastic carcinomas approximately equally account for 70 percent of the remainder of neoplasms.

TABLE 7.—Percentage distribution by histologic types of lung carcinomas observed at necropsy for men, according to time interval of death—A criteria

| Histologic type | 1927–34 | 1935–39 | 1940–44 | 1945–49 | 1950–54 | 1955–57 |
|---|---|---|---|---|---|---|
| | | All ages | | | | |
| | | All men | | | | |
| | | Number | | | | |
| All types | 34 | 81 | 143 | 152 | 185 | 134 |
| | | Percent of all types | | | | |
| | 100.0 | 100.0 | 100.1 | 100.0 | 99.9 | 99.9 |
| Adenocarcinoma | 2.9 | 7.4 | 6.3 | 14.5 | 13.5 | 10.4 |
| Epidermoid | 47.1 | 38.3 | 42.7 | 36.2 | 32.4 | 38.8 |
| Anaplastic | 41.2 | 44.4 | 29.4 | 35.5 | 32.4 | 32.8 |
| Mixed* | 5.9 | 7.4 | 10.5 | 3.9 | 3.8 | 6.7 |
| Other† | 2.9 | 2.5 | 11.2 | 9.9 | 17.8 | 11.2 |
| | | Caucasian men | | | | |
| | | Number | | | | |
| All types | 24 | 72 | 119 | 129 | 154 | 112 |
| | | Percent of all types | | | | |
| | 100.1 | 100.0 | 100.0 | 99.9 | 100.0 | 99.9 |
| Adenocarcinoma | 4.2 | 6.9 | 6.7 | 15.5 | 13.0 | 9.8 |
| Epidermoid | 54.2 | 40.3 | 42.9 | 36.4 | 33.1 | 40.2 |
| Anaplastic | 29.2 | 43.1 | 29.4 | 34.9 | 35.1 | 33.9 |
| Mixed* | 8.3 | 6.9 | 9.2 | 3.8 | 3.2 | 7.1 |
| Other† | 4.2 | 2.8 | 11.8 | 9.3 | 15.6 | 8.9 |

*Combined epidermoid and adenocarcinoma.

†Includes all other primary lung carcinomas reclassified histologically for this study (bronchiolar carcinomas, bronchial adenomas, and unclassified carcinomas).

TABLE 8.—Percentage distribution by histologic types of lung carcinomas observed at necropsy for women, according to time interval of death—A criteria

| Histologic types | All ages | 1927–44 | 1945–57 |
|---|---|---|---|
| | All women | | |
| | Number | | |
| All types | | 54 | 88 |
| | Percent of all types | | |
| | | 100. 0 | 100. 1 |
| Adenocarcinoma | | 11. 1 | 20. 5 |
| Epidermoid | | 35. 2 | 21. 6 |
| Anaplastic | | 14. 8 | 27. 3 |
| Mixed* | | 16. 7 | 5. 7 |
| Other† | | 22. 2 | 25. 0 |
| | Caucasian women | | |
| | Number | | |
| All types | | 39 | 50 |
| | Percent of all types | | |
| | | 99. 9 | 100. 0 |
| Adenocarcinoma | | 12. 8 | 24. 0 |
| Epidermoid | | 28. 2 | 18. 0 |
| Anaplastic | | 17. 9 | 28. 0 |
| Mixed* | | 23. 1 | 2. 0 |
| Other† | | 17. 9 | 28. 0 |

*Combined epidermoid and adenocarcinoma.

†Includes all other primary lung carcinomas reclassified histologically for this study (bronchiolar carcinomas, bronchial adenomas, and unclassified carcinomas).

The percentage of adenocarcinomas increases to approximately 30 percent when the B criteria are used for classification. This exceeds the proportion of either epidermoid or anaplastic carcinomas which, though still approximately equal in number, decreases to approximately 28 percent each. The minor histologic types account for the remainder.

The adenocarcinomas classified according to the B criteria are divisible into three histologic groups: 1) the differentiated adenocarcinomas, 2) adenocarcinomas with varying amounts of clusters of cohesive cells distributed in nests, which we arbitrarily refer to as cytocohesive adenocarcinoma, and 3) giant- and spindle-cell carcinomas. Our cytocohesive variants and giant- and spindle-cell adenocarcinomas conform to the large-cell adenocarcinoma described in the B criteria. Each of these distinct histologic groups shows differences in its relative proportions and time trends. These trends are shown in table 9. The differentiated adenocarcinomas are seen more frequently in the more recent time periods. The cytocohesive variants show periodic increases and decreases. The giant- and spindle-cell carcinomas, heretofore regarded as rare, have shown a consistent increase since 1940, and in the more recent time period these tumors constitute 15 percent of pulmonary carcinomas in men. The frequency of bronchiolar tumors is constant with either system of classification, their proportions varying between 1 and 3 percent.

TABLE 9.—Percentage distribution by histologic types of lung carcinomas observed at necropsy for men, according to time interval of death—B criteria

| Histologic type | All ages | | | | | |
|---|---|---|---|---|---|---|
| | 1927–34 | 1935–39 | 1940–44 | 1945–49 | 1950–54 | 1955–57 |
| **All men** | | | | | | |
| | Number | | | | | |
| All types | 35 | 81 | 142 | 158 | 196 | 147 |
| | Percent of all types | | | | | |
| | 100.1 | 100.0 | 100.0 | 100.0 | 100.1 | 100.1 |
| Adenocarcinoma | 11.4 | 25.9 | 25.4 | 24.1 | 29.6 | 36.1 |
| Differentiated | 2.9 | 9.9 | 8.5 | 12.7 | 15.3 | 11.6 |
| Cytocohesive | 8.6 | 7.4 | 11.3 | 3.8 | 2.6 | 9.5 |
| Giant and spindle-cell | — | 8.6 | 5.6 | 7.6 | 11.7 | 15.0 |
| Epidermoid | 42.9 | 30.9 | 35.9 | 34.8 | 32.7 | 30.6 |
| Anaplastic | 40.0 | 38.3 | 27.5 | 32.3 | 26.0 | 25.9 |
| Mixed* | 2.9 | 3.7 | 5.6 | 3.8 | 4.1 | 2.7 |
| Bronchiolar | — | 1.2 | 2.8 | 2.5 | 3.6 | 1.4 |
| Other† | 2.9 | — | 2.8 | 2.5 | 4.1 | 3.4 |
| **Caucasian men** | | | | | | |
| | Number | | | | | |
| All types | 25 | 72 | 118 | 134 | 165 | 122 |
| | Percent of all types | | | | | |
| | 100.0 | 100.0 | 99.9 | 100.0 | 99.9 | 100.0 |
| Adenocarcinoma | 16.0 | 23.6 | 25.4 | 27.6 | 28.5 | 34.4 |
| Differentiated | 4.0 | 6.9 | 8.5 | 14.2 | 13.3 | 10.7 |
| Cytocohesive | 12.0 | 8.3 | 10.2 | 4.5 | 3.0 | 10.7 |
| Giant and spindle-cell | — | 8.3 | 6.8 | 9.0 | 12.1 | 13.1 |
| Epidermoid | 48.0 | 31.9 | 36.4 | 33.6 | 32.1 | 31.1 |
| Anaplastic | 28.0 | 40.3 | 28.0 | 30.6 | 28.5 | 27.1 |
| Mixed* | 4.0 | 2.8 | 4.2 | 3.0 | 3.6 | 2.5 |
| Bronchiolar | — | 1.4 | 2.5 | 2.2 | 4.2 | 0.8 |
| Other† | 4.0 | — | 3.4 | 3.0 | 3.0 | 4.1 |

*Combined epidermoid and adenocarcinoma.

†Includes all other primary lung cancers reclassified histologically for this study (bronchial adenomas and unclassified carcinomas).

In women, differences in findings by criteria used affect both the proportions of individual histologic types and the trends of appearance. When neoplasms were classified by A criteria, as indicated in table 8, both adenocarcinomas and anaplastic carcinomas showed an increase, accompanied by a decrease of epidermoid tumors. Despite the high incidence of lung cancer in Mexican women (*8*, *9*), no difference in trends could be observed when the histologic patterns of this group were compared with those of Caucasians. When B criteria were used, an increase in adenocarcinoma was observed, though the other two groups remained virtually constant. The increase was accompanied by a decrease of the minor histologic types. The pattern for epidermoid and anaplastic carcinomas for Mexican and Caucasian women showed no increase, though the small numbers involved may be partially responsible for this

TABLE 10.—Percentage distribution by histologic types of lung carcinomas observed at necropsy for women, according to time interval of death—B criteria

| Histologic type | All ages | 1927–44 | 1945–57 |
|---|---|---|---|
| | **All women** | | |
| | Number | | |
| All types | | 52 | 91 |
| | Percent of all types | | |
| | | 99. 9 | 100. 0 |
| Adenocarcinoma | | 34. 6 | 53. 8 |
| Differentiated | | 9. 6 | 28. 6 |
| Cytocohesive | | 19. 2 | 8. 8 |
| Giant and spindle-cell | | 5. 8 | 16. 5 |
| Epidermoid | | 19. 2 | 18. 7 |
| Anaplastic | | 19. 2 | 18. 7 |
| Mixed* | | 7. 7 | 1. 1 |
| Bronchiolar | | 17. 3 | 3. 3 |
| Other† | | 1. 9 | 4. 4 |
| | **Caucasian women** | | |
| | Number | | |
| All types | | 37 | 52 |
| | Percent of all types | | |
| | | 100. 0 | 99. 9 |
| Adenocarcinoma | | 35. 1 | 59. 6 |
| Differentiated | | 10. 8 | 32. 7 |
| Cytocohesive | | 18. 9 | 3. 8 |
| Giant and spindle-cell | | 5. 4 | 23. 1 |
| Epidermoid | | 10. 8 | 17. 3 |
| Anaplastic | | 24. 3 | 19. 2 |
| Mixed* | | 10. 8 | — |
| Bronchiolar | | 18. 9 | 1. 9 |
| Other† | | — | 1. 9 |

*Combined epidermoid and adenocarcinoma.

†Includes all other primary lung carcinomas reclassified histologically for this study (bronchial adenomas and unclassified carcinomas).

observation. As in men, the proportion of adenocarcinomas is most significantly altered when the two criteria are compared, with a larger proportion being diagnosed when the B criteria were used. Analysis of adenocarcinomas classified by B criteria results in essentially the same pattern as that seen in men, as shown in table 10 and Appendix E. Independent of the criteria used, women have a higher proportion of adenocarcinomas than men. Nevertheless, an increased proportion of adenocarcinomas in both sexes resulted from the use of B criteria. Of the three subgroups of adenocarcinomas, the cytocohesive variant was responsible for the higher percentage of adenocarcinomas in women during 1927–44. There was an increase in only two subgroups during the recent inclusive time period, which, however, was most marked for differentiated adenocarcinomas. In the second inclusive time period the giant- and spindle-cell carcinomas have become a subgroup of increasing importance in women. This parallels the observations made in men.

TABLE 11.—Percentage distribution of lung carcinomas in men and women by locus and time interval of death

| Locus | Men | | Women | |
|---|---|---|---|---|
| | 1927–44 | 1945–57 | 1927–44 | 1945–57 |
| Major bronchi | 85. 1 | 82. 1 | 65. 0 | 72. 5 |
| Main stem R | 21. 3 | 18. 5 | 11. 7 | 13. 2 |
| L | 16. 2 | 14. 1 | 5. 0 | 13. 2 |
| Lobar and segmental | | | | |
| Upper R | 10. 8 | 13. 9 | 5. 0 | 13. 2 |
| L | 10. 8 | 15. 9 | 8. 3 | 16. 5 |
| Lower R | 10. 8 | 8. 3 | 6. 7 | 6. 6 |
| L | 5. 1 | 5. 7 | 6. 7 | 5. 5 |
| Middle R | 6. 1 | 4. 9 | 11. 7 | 2. 2 |
| Not stated | 4. 1 | 0. 8 | 10. 0 | 2. 2 |
| Periphery | 8. 4 | 12. 8 | 16. 7 | 18. 7 |
| Multiple sites | 1. 4 | 2. 4 | 10. 0 | 5. 5 |
| Not stated | 5. 1 | 2. 8 | 8. 3 | 3. 3 |
| Total percent | 100. 0 | 100. 1 | 100. 0 | 100. 0 |
| Number | (296) | (509) | (60) | (91) |

*Diagnoses in relation to locus.*—At necropsy, especially in the advanced stages, it is frequently impossible to determine the exact site of tumor origin, and only the location of the main tumor mass can be established. The distribution of neoplasms by locus is given in table 11. In men, main stem, lobar, and segmental bronchi are the most common sites. The pattern in women varies for the two time periods, probably because of the small numbers involved. In the most recent period, however, the distribution is essentially similar to that observed in men. A comparison of our findings with those of Olson (*32*), and Bryson and Spencer (*33*) is shown in table 12. Although the data are essentially similar, certain inconsistencies may be observed. We have found fewer left lower-lobe tumors and more middle-lobe tumors than were noted in the other studies.

The relation of histologic type to locus with the B criteria is given in table 13. Only bronchiolar tumors are restricted exclusively to a periph-

TABLE 12.—Percentage distribution by locus of lung carcinomas as observed at necropsy in bronchi*

| Locus | Present study | Olson | Bryson and Spencer |
|---|---|---|---|
| Major bronchi | | | |
| Main stem R | 21. 7 | 17. 6 | 17. 6 |
| L | 16. 6 | 8. 8 | 17. 6 |
| Lobar and segmental | | | |
| Upper R | 14. 5 | 19. 1 | 15. 2 |
| L | 16. 4 | 19. 1 | 18. 7 |
| Lower R | 10. 3 | 8. 8 | 13. 9 |
| L | 6. 5 | 17. 6 | 11. 2 |
| Middle R | 6. 4 | 2. 9 | 2. 6 |
| Not stated | 7. 5 | 5. 9 | 3. 2 |
| Total percent | 99. 9 | 99. 8 | 100. 0 |
| Number | (812) | (68) | (841) |

*Excludes peripheral and multiple tumors. *Note* differences from findings of Olson (*32*) and Bryson and Spencer (*33*).

eral site in the lung. Epidermoid and anaplastic carcinomas, though seen throughout the lung, were concentrated in the main stem, lobar, and segmental bronchi. Adenocarcinomas were also located more frequently in main stem, lobar, and segmental bronchi. However, a higher proportion of adenocarcinomas was more frequently peripheral in site than were either anaplastic or epidermoid, though the proportion of these does not equal that reported in the literature (*34*, *35*). The relative paucity of peripheral adenocarcinomas in our series may reflect the extension of the tumor into a major bronchus as seen at necropsy. No differences between women and men were observed when locus was related to histologic type.

TABLE 13.—Percentage distribution by locus of histologic type of lung carcinomas for men and women—B criteria

| Locus | Adenocarcinoma | Epidermoid | Anaplastic | Mixed | Bronchiolar | Other* | Total |
|---|---|---|---|---|---|---|---|
| | | | | Men | | | |
| | | | | Percent of all loci | | | |
| Bronchi† | 72.9 | 93.7 | 91.1 | 76.7 | — | 75.0 | 83.1 |
| Periphery | 20.5 | 4.3 | 5.8 | 20.0 | 50.0 | 11.8 | 11.2 |
| Multiple | 1.4 | .4 | .4 | 3.3 | 50.0 | 1.5 | 2.0 |
| Not stated | 5.2 | 1.6 | 2.7 | — | — | 11.8 | 3.6 |
| All loci (percent) | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.1 | 99.9 |
| Number | (210) | (255) | (224) | (30) | (18) | (68) | (805) |
| | | | | Women | | | |
| Bronchi† | 67.2 | 92.6 | 88.9 | 80.0 | — | 53.8 | 69.5 |
| Periphery | 20.9 | 7.4 | 7.4 | — | 41.7 | 30.8 | 17.9 |
| Multiple | 4.5 | — | 3.7 | 20.0 | 58.3 | — | 7.9 |
| Not stated | 7.5 | — | — | — | — | 15.4 | 4.6 |
| All loci (percent) | 100.1 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 99.9 |
| Number | (67) | (27) | (27) | (5) | (12) | (13) | (151) |

*Includes all other primary lung carcinomas reviewed for this study.
†Includes main stem, lobar, and segmental bronchi.

*Diagnoses in relation to age.*—The frequency of all histologic types of lung cancer by age in men was greatest in the middle-age period (45–64 years). Adenocarcinomas did not appear in significantly greater proportions in the 65-and-over age group than did the other two types. This is contrary to findings of Kreyberg (*5*) and others. In women, there was a perceptible increase in adenocarcinomas in the 65-and-over age group. Both epidermoid and adenocarcinomas were observed more frequently in women than in men in the under-45 age group (table 14).

## DISCUSSION

For convenience and clarity, the discussion of the data is divided into two main sections: The first deals with the histologic phase and the

TABLE 14.—Age distribution of histologic types of lung carcinoma for men and women—A and B criteria

| | Men | | | | | Women | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | All ages | | | | | All ages | |
| Histologic type | <45 | 45–64 | 65+ | Percent | No. | <45 | 45–64 | 65+ | Percent | No. |
| A Criteria | | | | | | | | | | |
| All types | 6.7 | 55.4 | 37.9 | 100.0 | 728 | 11.3 | 43.7 | 45.1 | 100.1 | 142 |
| Adenocarcinoma | 6.5 | 51.9 | 41.6 | 100.0 | 76* | 20.8 | 29.2 | 50.0 | 100.0 | 24 |
| Epidermoid | 4.7 | 57.1 | 38.2 | 100.0 | 275 | 15.8 | 47.4 | 36.8 | 100.0 | 38 |
| Anaplastic | 7.6 | 58.0 | 34.4 | 100.0 | 250 | 9.4 | 56.3 | 34.4 | 100.1 | 32 |
| Others† | 9.4 | 48.8 | 41.7 | 99.9 | 127 | 4.2 | 39.6 | 56.3 | 100.1 | 48 |
| B Criteria | | | | | | | | | | |
| All types | 6.7 | 55.4 | 37.9 | 100.0 | 758 | 11.2 | 43.4 | 45.5 | 100.1 | 143 |
| Adenocarcinoma | 6.7 | 54.1 | 39.2 | 100.0 | 209* | 13.4 | 38.8 | 47.8 | 100.0 | 67 |
| Epidermoid | 4.3 | 57.6 | 38.0 | 100.0 | 255 | 18.5 | 48.1 | 33.3 | 99.9 | 27 |
| Anaplastic | 8.0 | 56.3 | 35.7 | 100.0 | 224 | 7.4 | 63.0 | 29.6 | 100.0 | 27 |
| Others† | 11.4 | 48.6 | 40.0 | 100.0 | 70 | — | 27.3 | 72.7 | 100.0 | 22 |

*Excludes one male of unknown age.

†Includes all other primary lung carcinomas reclassified histologically for this study (bronchiolar carcinomas, unclassified carcinomas, bronchial adenomas, and combined epidermoid and adenocarcinomas).

second with the statistical and epidemiologic implications of morphologic findings.

## Histologic Studies

Before relating the histologic findings of pulmonary neoplasms in our series to the increasing incidence of this disease, we need to comment on certain specific aspects of our study. First, the effect of the use of two sets of criteria in the interpretation of our material must be examined. Second, differences arising from the use of frozen-tissue sections and paraffin-block sections need explanation. Third, the effect on the ultimate diagnoses resulting from differences in the interpretation by the author and the various consultants should be evaluated. It is only after the effect of the several factors on the diagnosis has been determined that we can attempt to establish any etiologic implications of our data.

*Differences resulting from the use of A and B criteria.*—The A criteria, aimed at simplicity, appeared to have limitations in the anaplastic carcinoma group, because the small- and large-cell carcinomas were placed in a single category and their biologic behavior requires a distinction. These groups, however, were separated into two distinct categories when the B criteria were used. Because of the small proportion of large-cell anaplastic or undifferentiated carcinomas in our series, we combined them with the small-cell carcinomas (fig. 23) in some of our tabulations (tables 9, 13, 14, and Appendix).

Approximately 30 percent of lung neoplasms were large-cell, poorly differentiated, and undifferentiated carcinomas morphologically. These heterogeneous groups, including our giant- and spindle-cell variants, were difficult to classify on hematoxylin-and-eosin-stained sections alone; however, they could be subdivided into several categories, as shown in table

6. Originally, we regarded neoplasms having nests of cohesive tumor cells or containing large and/or giant cells with abundant eosinophilic cytoplasm as epidermoid carcinoma (figs. 2, 3, 9, 10, 11, 12, 14, and 19). This concept, though currently in use in the United States, is probably an erroneous one, resulting from a misapplication of the criteria of Liebow (*18, 21, 27–30*).

The heretofore uncommonly diagnosed giant- and spindle-cell carcinomas were observed frequently in our series (figs. 1, 18, 20, and 22). Hadley and Bullock (*36*) reported that these tumors represented 9 percent of the lung neoplasms diagnosed at necropsy at the Los Angeles County Hospital in 1951. They classified these neoplasms as a subgroup of anaplastic carcinoma, as did we, when using A criteria. Nash and Stout (*37*) reported finding a similar neoplasm which they regarded as a histologic variant of primary lung carcinoma, either undifferentiated epidermoid or adenocarcinoma.

More finite classification of pulmonary neoplasms, especially in the poorly differentiated and undifferentiated group, resulted from the use of B criteria. This distinction is understandable when we consider that for the A criteria we utilized hematoxylin-and-eosin-stained material and the classification was on morphologic characteristics alone. In marked contrast, the application of the B criteria was predicated on more rigid class definitions and on the use of histochemical reactions in addition to morphologic observations for the poorly differentiated and undifferentiated neoplasms. Tumors in this group in which intracytoplasmic mucopolysaccharide was demonstrable were classified as adenocarcinoma. As a result, an increase in the adenocarcinoma group and a corresponding decrease in the epidermoid, anaplastic, and mixed carcinoma were observed (figs. 7 and 8). The large-cell undifferentiated carcinoma subgroup was reduced to a mere 1 percent. Kreyberg classified most of the tumors, similar to those listed in table 6, as large-cell adenocarcinomas.

Our data suggesting that the increased incidence of pulmonary carcinoma is largely characterized by an increase in the adenocarcinoma group vary with those of other investigators (*1–4, 10, 13, 31*). The differences may be largely due to at least two factors: individual interpretation of various pathologists, and perhaps more significantly, the histochemical stains used in preparation of the tissue. On the basis of routine hematoxylin and eosin sections, Hadley and Bullock (*36*) found 13 percent of all primary pulmonary neoplasms observed at necropsy at the Los Angeles County Hospital for 1951 to be adenocarcinomas. This agrees with the 10.6 percent adenocarcinomas we found for the entire series, when using A criteria. Spain and O'Neal *et al.*, in studies limited to routine hematoxylin and eosin sections, classified 31 and 20 percent, respectively, of their series as adenocarcinomas. Spain emphasized that the relative frequency of adenocarcinoma decreased with the passage of time. However, some of the early percentages reported by both Spain and O'Neal are based on few necropsies, and the reliability may be questionable. Gibson (*38*), using routine hematoxylin and eosin sections, found that 38

percent of 120 primary lung carcinomas found at necropsy at the University of Kansas Medical Center from 1914–49 were adenocarcinomas. He used strict criteria in the diagnosis of epidermoid and adenocarcinomas.

The proportion of large-cell undifferentiated or anaplastic carcinomas also varies considerably among studies. Hadley and Bullock (*36*) found 20 percent undifferentiated large-cell carcinomas and 9 percent giant- and spindle-cell carcinomas among the lung carcinomas observed at necropsy at the Los Angeles County Hospital in 1951. As noted previously, 30 percent of our pulmonary neoplasms, including the giant- and spindle-cell variant, were large-cell, poorly differentiated, and undifferentiated carcinomas. When further classified by the use of A criteria, 15 percent of the total series were placed in an unclassified category, 5 percent were designated as either large-cell anaplastic (figs. 4 and 17) or anaplastic giant- and spindle-cell carcinomas (figs. 1, 18, 20, and 22), and 10 percent were erroneously classified as epidermoid carcinomas [cytocohesive (figs. 3, 9, 10, 11, and 12) or giant-cell type (figs. 2, 14, and 19)], or mixed epidermoid and adenocarcinoma (figs. 5, 15, and 16). O'Neal *et al.* (*10*), using the Kirklin and McDonald method (*39*) to analyze autopsy material, classified 34.9 percent of their bronchogenic tumors as large-cell anaplastic carcinomas in a series of 301. Adequate comparisons of our findings with those of Spain are impossible, since he classified large and small anaplastic carcinomas together with epidermoid carcinomas.

With the use of B criteria, our large-cell undifferentiated and giant- and spindle-cell carcinomas, unclassified tumors, mixed carcinomas, and epidermoid carcinomas all decreased, while the adenocarcinomas increased from 10 to 29 percent of all lung tumors. These findings partially agree with those of Walter and Pryce (*35*) in the instance of adenocarcinomas. They reported that 28.3 percent of 159 lung tumors observed at necropsy was adenocarcinoma and 10.7 percent was polygonal-cell carcinoma. This latter group conforms to our category of large-cell anaplastic or undifferentiated carcinoma. Although their morphologic classification differs from ours, their method of study was similar in that they used special stains for demonstrating mucin. They observed giant-cell formation in some of their neoplasms and considered this a secondary change which could occur in all tumors independent of cell type. We concur with the concept that neoplastic giant cells can be found in all lung carcinomas. However, in our series, a majority of tumors composed predominantly of giant and/or spindle cells were shown to contain intracytoplasmic acid mucopolysaccharide and on this basis were designated as a subgroup of adenocarcinoma. In addition, neoplastic giant and spindle cells were noted in varying numbers in some epidermoid carcinomas (figs. 6 and 13), anaplastic small-cell carcinomas, metastatic sarcomas (fibrosarcoma, liposarcoma, and myosarcoma), metastatic melanomas, and several unclassified lung tumors. This cell pattern was especially prominent in neoplasms that had previously been treated with X-ray therapy or chemotherapy.

We believe that a portion of neoplasms classified by other investigators

as large-cell undifferentiated, epidermoid (our cytocohesive variant or giant-cell variant), and anaplastic giant-cell carcinomas on hematoxylin and eosin sections probably will be found to contain acid mucopolysaccharide when appropriate stains are used.

*Effect of frozen section and paraffin sections on classification.*—The recognized limitations of frozen sections when compared with paraffin sections probably account for some of the differences in histologic diagnosis that exist in a portion of the neoplasms. When using A criteria, we found more mixed tumors in both sexes during the first inclusive time period than the second. When using B criteria, we diagnosed bronchiolar carcinoma in women with decreasing frequency during the second inclusive time period. The improved histologic sections resulting from use of paraffin technique combined with the increased number of individual sections possible from each tumor may account for this observation. Occasionally, additional tissue sections revealed involvement of a large bronchus by papillary adenocarcinoma, and thus required a change in the diagnosis from bronchiolar carcinoma to adenocarcinoma.

*Effect of change in criteria on diagnosis.*—More definite, though possibly less reliable, histologic diagnoses were made on the hematoxylin-and-eosin-stained frozen sections than for the paraffin sections, since during the classification of the frozen-section material we were familiar with and utilized only A criteria. This limited approach, while permitting histologic classification of more tumors, regrettably did not as a corollary make for more accurate diagnoses. While evaluating the paraffin material, we learned about the criteria described under B and readily recognized that the histologic reclassification of numerous neoplasms was indicated. In view of this, we altered the method of designation when using A criteria, and more paraffin-sectioned tumors stained with hematoxylin and eosin were classified as uncertain. The altered diagnoses resulting from introduction of the second or B criteria produced changes in histologic interpretation which were also based on rigid adherence to the criteria.

Sections from 100 neoplasms in our series were randomly selected and sent to Kreyberg for diagnosis, inasmuch as the WHO criteria and method of classification to a large degree reflect his efforts. In general, we were in consistent agreement with Kreyberg's diagnoses when we classified our material according to the B criteria. Differences were limited to carcinoid tumors, which we diagnosed to a lesser extent, and mixed epidermoid and adenocarcinomas (figs. 7 and 8), which we diagnosed to a greater extent. From a broad viewpoint, we feel that our method of study and application of B criteria are comparable to those Kreyberg used in analyzing his data from Norway. If this is so, the increasing incidence of adenocarcinoma in our series, which is in marked contrast to the decreasing incidence in his necropsy series, is in need of explanation. Certainly, such environmental factors as geographic location, occupational differences, variations in quality and quantity of air pollutants, and socioeconomic factors must be considered.

## Statistical Studies

Independent of the criteria used, our observations differ in varying degrees from similar studies reported in the literature.

### *A Criteria*

*Ratio of epidermoid plus undifferentiated (anaplastic small and large cell) carcinomas to adenocarcinoma.*—When the A criteria, as devised by Liebow (*18*), were used, the data obtained were compared with those of Spain (*11*) and O'Neal *et al.* (*10*) in terms of the ratio of epidermoid plus undifferentiated carcinomas (group 1) to adenocarcinomas (group 2) as shown in table 15. Comparisons of our findings with data reported by Spain and O'Neal *et al.* may not be completely valid for two reasons: 1) The studies differed in that ours included all carcinomas of the lung, while theirs covered only bronchogenic carcinomas, and 2) our designation of mixed epidermoid and adenocarcinoma as a distinct category is in contrast to O'Neal *et al.* (*10*) who excluded one mixed tumor from their series, and Spain (*11*) who "included mixed patterns that contained acini" with the adenocarcinoma group. Because of variations among these studies, we computed our ratios of group 1 to group 2 tumors with group 2 tumors including only adenocarcinoma and also with group 2 tumors including both adenocarcinoma and mixed epidermoid and adenocarcinoma. The data of both Spain and O'Neal *et al.* show a slight increase in the ratio of group 1 to group 2 tumors during the last 25 years. The number of tumors in their series for the earlier years is too small to justify an analysis (*see* Appendix F). Our data for men, in contrast, show a sharp decrease in the ratio at the fourth time period, with the maintenance of a fairly constant ratio since then. This sharp decline that occurred at the fourth time period coincided with the innovation of paraffin sections. The trend is more apparent when computed on the basis of adenocarcinomas alone. Our high ratios for the first two time periods are unreliable because of the occurrence of only a few adenocarcinomas. For the most recent time period our ratio of 6.7 to 1 (computed for adenocarcinomas alone) is comparable to that of O'Neal, while our ratio of 4.2 to 1 (computed for adenocarcinomas plus mixed tumors) is close to that of Spain's. An excess of epidermoid and undifferentiated carcinomas is reported in all three studies.

Percentages of each type of lung tumor relative to all autopsies give further information on the changes in frequency of each type, provided there have not been radical changes in the characteristics of the necropsy populations over the years. These percentages are available for the Spain and O'Neal *et al.* data for each sex (Appendix F) and for both sexes combined for the present study (table 16). The slightly increasing ratios reported by O'Neal mirror a high proportion of undifferentiated and epidermoid carcinomas from 1930–54. The low ratios reported for the years before 1929 are based on small numbers and are less reliable. In data reported by Spain, the low ratios for the early time period are also obtained from too few necropsies to be reliable. The ratios and per-

TABLE 15.—Ratios of epidermoid plus undifferentiated carcinomas to adenocarcinomas (A criteria) in relation to time and sex. Data are compared with those of O'Neal *et al.*, and Spain to show differences

| Present study | | | | | O'Neal *et al.* (*10*) | | | Spain (*11*) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Men | | Women | | | Men | Women | | Men | Women |
| Year | Ratio (1)*/(2)† | Ratio (1)*/(2)‡ | Ratio (1)*/(2)† | Ratio (1)*/(2)‡ | Year | Ratio (1)*/(2)† | Ratio (1)*/(2)† | Year | Ratio (1)*/(2)† | Ratio (1)*/(2)† |
| — | — | — | — | — | 1910–19 | (—)§ | (—) | 1912–21 | (0. 7) | (0) |
| 1927–34 | 30. 0 | 10. 0 | (3. 5) | 2. 3 | 1920–29 | (2. 0) | (0. 3) | 1922–31 | 3. 0 | (0) |
| 1935–39 | 11. 2 | 5. 6 | 1. 5 | 0. 7 | 1930–39 | 3. 9 | (1. 0) | 1932–41 | 2. 6 | 0. 6 |
| 1940–44 | 11. 4 | 4. 3 | — | 4. 7 | 1940–44 | 6. 4 | (6. 0) | 1942–46 | 3. 4 | (0. 8) |
| 1945–49 | 5. 0 | 3. 9 | 2. 3 | 1. 8 | 1945–54 | 5. 6 | 1. 1 | 1947–56 | 3. 8 | 1. 5 |
| 1950–54 | 4. 8 | 3. 7 | 2. 3 | 2. 0 | | | | | | |
| 1955–57 | 6. 7 | 4. 2 | 2. 5 | 1. 8 | | | | | | |

*(1) Epidermoid and undifferentiated carcinoma.
†(2) Adenocarcinoma.
‡(2) Adenocarcinoma and mixed epidermoid and adenocarcinoma.
§Ratios in parentheses are based on a total of less than 10 necropsies.

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TABLE 16.—Comparisons of total epidermoid plus undifferentiated (anaplastic small- and large-cell carcinomas) with adenocarcinomas, mixed epidermoid and adenocarcinomas (A criteria) and all primary lung carcinomas for sequential time intervals

| Year of death | Epidermoid plus undifferentiated | | Adenocarcinoma* | | Mixed epidermoid and adenocarcinoma* | | Adenocarcinoma* plus mixed epidermoid and adenocarcinoma* | | All lung carcinomas | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Number | Percent all necropsies | Number | Percent all necropsies | Number | Percent all necropsies | Number | Percent all necropsies | Number | Percent all necropsies |
| 1927–34 | 37 | (0. 4) | 3 | (0. 03)† | 3 | (0. 03)† | 6 | (0. 06)† | 53 | (0. 6) |
| 1935–39 | 73 | (0. 7) | 10 | (0. 1) | 11 | (0. 1) | 21 | (0. 2) | 111 | (1. 1) |
| 1940–44 | 117 | (1. 2) | 9 | (0. 1)† | 18 | (0. 2) | 27 | (0. 3) | 192 | (1. 9) |
| 1945–49 | 118 | (1. 3) | 26 | (0. 3) | 7 | (0. 07)† | 33 | (0. 4) | 184 | (2. 1) |
| 1950–54 | 136 | (1. 4) | 32 | (0. 3) | 8 | (0. 1)† | 40 | (0. 4) | 229 | (2. 4) |
| 1955–57 | 114 | (1. 6) | 22 | (0. 3) | 11 | (0. 2) | 33 | (0. 5) | 187 | (2. 6) |
| Total | 595 | (1. 1) | 102 | (0. 2) | 58 | (0. 1) | 160 | (0. 3) | 956 | (1. 7) |

*Excludes bronchiolar tumors.
†Percentage based on a total of less than 10 necropsies.

centages reported by Spain have remained relatively constant since 1922. In the present study, both groups of tumors have shown an increase relative to all necropsies. Neither the undifferentiated and epidermoid group nor the adenocarcinoma group has shown a disproportionate increase when classified according to the A criteria, and both groups of tumors have contributed to the substantial rise in total lung carcinomas observed at necropsy. The mixed epidermoid and adenocarcinoma group has fluctuated, showing a decrease at the fourth time period, when paraffin-section technique was instituted. In all three studies, lung carcinomas have been increasing in proportion to all necropsies, but this increase by the various histologic types is not the same in each study.

Direct comparisons of individual ratios or proportions in the three studies are impossible because of differences in the characteristics of the populations used as bases for computation. For example, our data are computed on the basis of the entire autopsy population, while the other two studies refer only to the adult population. This explains, in part, the low proportion of lung tumors in the Los Angeles County Hospital material. A second factor perhaps accounting for the minor differences reported is the high proportions of Mexican and Negro patients (close to 20 and 33%, respectively, in 1957) (*40*) treated at the Los Angeles County Hospital. Further, the older population group probably comprises a larger proportion than found in the hospital population reported by Spain and by O'Neal *et al.* While neither race nor age appears closely linked to histologic type (Appendix A, B, C, and D), they may conceivably be a factor in distinguishing our data from those of other sources.

The effect of socioeconomic factors in the Los Angeles County Hospital may be of greater importance. The population treated at the hospital is primarily an economically indigent one. While nothing is known of the effect of economic status on tumor morphology, both Cohart (*41*) and the Registrar-General in Great Britain (*42*) have reported an inverse relationship between socioeconomic status and lung cancer in general. Both indicate an increased incidence in persons of low socioeconomic level.

The number of lung carcinomas in women is small in all three studies, and temporal trends are difficult to assess. The ratios in all the studies are close to 1 or 2. In recent years, there has been a preponderance of undifferentiated and epidermoid carcinomas, but it is not as great as that noted for men. The Los Angeles County Hospital material shows a greater excess than the other two studies. The higher proportion of Mexican women included in the Los Angeles County Hospital data, however, probably does not account for this greater number.

### *B Criteria*

Comparison of data classified by B criteria is possible only with the findings reported by Jakobsen, Christiansen, and Kreyberg (*1–5*) (table 17). Because of the restricted nature of the data from Norway, we abstracted only data on Caucasians for comparison. The Norwegian study reveals a temporal increase in the ratio of Group I (epidermoid,

anaplastic small-cell, and undifferentiated large-cell carcinomas) to Group II tumors [7] (adenocarcinomas, bronchial adenomas, bronchiolar, and mixed epidermoid and adenocarcinomas), with twofold to threefold excess of the former. Our data, as noted, reveal a decrease in the ratio with the passage of time with a ratio of approximately 1.5 in recent years. No data for the necropsy populations are given in the studies from Norway.

TABLE 17.—Comparisons of findings from present study with data from Norway (B criteria) by distribution of Group I to Group II lung carcinomas observed at necropsy for men and women by sequential time period*

| | Present study Caucasian men | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Year of death | Group I | Group II† | Ratio I/II | Study | Year of death | Group I | Group II | Ratio I/II |
| 1927–34 | 19 | 4 | 4. 8 | Christiansen | 1925–44 | 25 | 15 | 1. 7 |
| 1935–39 | 52 | 18 | 2. 7 | Jakobsen | 1937–46 | 46 | 24 | 1. 9 |
| 1940–44 | 76 | 34 | 2. 2 | Christiansen | 1945–52 | 40 | 14 | 2. 9 |
| 1945–49 | 86 | 41 | 2. 1 | | | | | |
| 1950–54 | 100 | 57 | 1. 8 | | | | | |
| 1955–57 | 71 | 44 | 1. 6 | | | | | |
| Total | 404 | 197 | 2. 1 | | | | | |
| | Caucasian women | | | | | | | |
| 1927–34 | 1 | 5 | 0. 2‡ | Christiansen | 1925–44 | 11 | 13 | 0. 9 |
| 1935–39 | 1 | 8 | 0. 1‡ | Jakobsen | 1937–46 | 8 | 22 | 0. 4 |
| 1940–44 | 10 | 7 | 1. 4 | Christiansen | 1945–52 | 4 | 12 | 0. 3 |
| 1945–49 | 5 | 7 | 0. 7 | | | | | |
| 1950–54 | 6 | 12 | 0. 5 | | | | | |
| 1955–57 | 8 | 14 | 0. 6 | | | | | |
| Total | 31 | 53 | 0. 6 | | | | | |

*See reference (5).
†Excludes mixed tumors; includes bronchiolar tumors and adenomas.
‡Ratios are based on a total of less than 10 necropsies.

The higher proportion of adenocarcinomas for women in our study and in the Norwegian material is reflected by the smaller ratios of Group I to Group II tumors. Time trends are based on small numbers of cases and must be interpreted in this light. The Norway ratios have shown a decrease, while the Los Angeles ratio has been fairly constant in recent years.

The distribution of all lung tumors for our data and for those of the data from Norway is compared in table 18. For men, the data are similar for all major types. Differences are noted in the proportions of large-cell carcinomas, and the adenoma-salivary-gland tumors in which a lower proportion for each can be noted in our figures. Tumor distributions for women differ in these two groups, though the high incidence of adenocarcinomas is evident in all the studies. Again, while the number of cases is small, we have more bronchiolar, mixed epidermoid and adenocarcinoma,

[7] For this comparison we excluded mixed epidermoid and adenocarcinomas in our series from the Group II tumors, since Kreyberg designated few, if any, neoplasms as mixed tumors in his necropsy series from Norway.

TABLE 18.—Distribution of lung carcinomas observed at necropsy by histologic type—B criteria. Data are compared with those of Jakobsen and Christiansen to show differences

| Histologic type | Present study* 1927–54 | Jakobsen (*1*) 1937–46 | Christiansen (*2*) 1925–52 |
|---|---|---|---|
| | (Percent of lung necropsies) | | |
| | Men | | |
| All types | | | |
| Number | 512 | 70 | 94 |
| Percent | 100. 1 | 100. 1 | 99. 9 |
| Adenocarcinoma | 26. 4 | 21. 4 | 19. 1 |
| Epidermoid | 34. 4 | 22. 9 | 31. 9 |
| Large cell, undifferentiated | 1. 4 | 10. 0 | 7. 4 |
| Small cell, anaplastic | 29. 3 | 32. 9 | 29. 8 |
| Bronchiolar | 2. 7 | 2. 9 | 4. 3 |
| Adenoma-salivary-gland tumors | 1. 0 | 10. 0 | 7. 4 |
| Others† | 4. 9 | — | — |
| | Women | | |
| All types | | | |
| Number | 67 | 30 | 40 |
| Percent | 100. 0 | 99. 9 | 100. 0 |
| Adenocarcinoma | 47. 8 | 50. 0 | 52. 5 |
| Epidermoid | 14. 9 | 10. 0 | 22. 5 |
| Large cell, undifferentiated | — | 13. 3 | 5. 0 |
| Small cell, anaplastic | 20. 9 | 3. 3 | 10. 0 |
| Bronchiolar | 10. 4 | — | 2. 5 |
| Adenoma-salivary-gland tumors | — | 23. 3 | 7. 5 |
| Others† | 6. 0 | — | — |

*Excludes Mexicans and non-Caucasians.

†Includes all other primary lung carcinomas reclassified histologically for this study (combined epidermoid and adenocarcinoma and unclassified carcinomas).

and small-cell carcinomas, and fewer large-cell carcinomas and adenoma-salivary-gland tumors.

## CONCLUSION

Our use of two systems of classification emphasizes certain patterns that are more obvious when the WHO method is used. Our ratios of epidermoid and undifferentiated carcinomas to adenocarcinomas for men are decreasing, while ratios in similar studies have shown an increase. The preponderance of epidermoid and undifferentiated carcinomas in males is clear in all the studies, but, while some United States reports and the Norway data indicate a disproportionate increase in these tumors over the years, the Los Angeles County Hospital population apparently may have reached a high incidence in earlier years.

The Los Angeles material is different from that reported elsewhere, but whether this discrepancy is caused by variations in autopsy populations or due to true differences in incidence cannot be determined by the material presented here. The dual classification of this material has given an indication that some of the differences in American and Norwegian studies can be attributed to variations in the criteria. Further studies in

APPENDIX A.—Distribution by histologic types of lung carcinomas observed at necropsy for men according to time interval of death—A criteria

| Histologic type | All men | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | All ages | | | | | | Ages 45–64 | | | | | | Ages 65+ | | | | | |
| | 1927–34 | 1935–39 | 1940–44 | 1945–49 | 1950–54 | 1955–57 | 1927–34 | 1935–39 | 1940–44 | 1945–49 | 1950–55 | 1955–57 | 1927–34 | 1935–39 | 1940–44 | 1945–49 | 1950–54 | 1955–57 |
| Adenocarcinoma | 1 | 6 | 9 | 22 | 25 | 14 | — | 4 | 6 | 14 | 10 | 5 | 1 | 2 | 3 | 7 | 11 | 8 |
| Epidermoid | 16 | 31 | 61 | 55 | 60 | 52 | 11 | 25 | 35 | 35 | 27 | 24 | 5 | 5 | 20 | 17 | 32 | 26 |
| Anaplastic | 14 | 36 | 42 | 59 | 60 | 44 | 10 | 22 | 27 | 28 | 33 | 25 | 1 | 10 | 10 | 22 | 25 | 18 |
| Mixed* | 2 | 6 | 15 | 6 | 7 | 9 | 2 | 4 | 11 | 2 | 3 | 4 | — | 2 | 4 | 4 | 3 | 4 |
| Other† | 1 | 2 | 16 | 15 | 33 | 15 | — | — | 8 | 9 | 11 | 8 | — | — | 7 | 5 | 17 | 7 |
| Total | 34 | 81 | 143 | 152 | 185 | 134 | 23 | 55 | 87 | 88 | 84 | 66 | 7 | 19 | 44 | 55 | 88 | 63 |
| | Caucasian men | | | | | | | | | | | | | | | | | |
| Adenocarcinoma | 1 | 5 | 8 | 20 | 20 | 11 | — | 4 | 5 | 12 | 9 | 4 | 1 | 1 | 3 | 7 | 8 | 6 |
| Epidermoid | 13 | 29 | 51 | 47 | 51 | 45 | 9 | 23 | 27 | 30 | 20 | 22 | 4 | 5 | 18 | 15 | 30 | 22 |
| Anaplastic | 7 | 31 | 35 | 45 | 54 | 38 | 6 | 17 | 22 | 25 | 30 | 21 | — | 10 | 9 | 18 | 23 | 16 |
| Mixed* | 2 | 5 | 11 | 5 | 5 | 8 | 2 | 3 | 8 | 1 | 2 | 4 | — | 2 | 3 | 4 | 2 | 3 |
| Other† | 1 | 2 | 14 | 12 | 24 | 10 | — | — | 6 | 9 | 9 | 5 | — | — | 7 | 3 | 13 | 5 |
| Total | 24 | 72 | 119 | 129 | 154 | 112 | 17 | 47 | 68 | 77 | 70 | 56 | 5 | 18 | 40 | 47 | 76 | 52 |

*Combined epidermoid and adenocarcinoma.
†Includes all other primary lung carcinomas reclassified histologically for this study.

this country, in which subgroups like the giant- and spindle-cell carcinomas will be investigated with the B criteria, would be valuable in the determination of whether the results presented here also exist in other areas.

The unanticipated results of our study place added emphasis on any etiologic implications related to our morphologic findings. Unfortunately, diligent scrutiny of occupational and general environmental histories reveals that data in relation to these areas are either absent or sparse and their reliability is questionable. Currently 225 cases are being studied in a modified prospective manner. In these patients, environmental and smoking histories are being obtained. Any possible relationships between morphologic and etiologic factors must await the final analyses of these cases. In the event, as now seems likely, that the trends reported are to continue, conclusions drawn from the prospective study will be examined for their possible application to this retrospective study.

APPENDIX B.—Distribution by histologic types of lung carcinomas observed at necropsy for women according to time interval of death—A criteria

| | All women | | | | | |
|---|---|---|---|---|---|---|
| | All women | | Ages 45–64 | | Ages 65+ | |
| Histologic type | 1927–44 | 1945–57 | 1927–44 | 1945–57 | 1927–44 | 1945–57 |
| Adenocarcinoma | 6 | 18 | 3 | 4 | 1 | 11 |
| Epidermoid | 19 | 19 | 11 | 7 | 5 | 9 |
| Anaplastic | 8 | 24 | 4 | 14 | 2 | 9 |
| Mixed* | 9 | 5 | 4 | 2 | 4 | 3 |
| Other† | 12 | 22 | 4 | 9 | 8 | 12 |
| Total | 54 | 88 | 26 | 36 | 20 | 44 |
| | Caucasian women | | | | | |
| Adenocarcinoma | 5 | 12 | 3 | 3 | 1 | 8 |
| Epidermoid | 11 | 9 | 7 | 4 | 4 | 3 |
| Anaplastic | 7 | 14 | 3 | 9 | 2 | 4 |
| Mixed* | 9 | 1 | 4 | — | 4 | 1 |
| Other† | 7 | 14 | 1 | 5 | 6 | 8 |
| Total | 39 | 50 | 18 | 21 | 17 | 24 |

*Combined epidermoid and adenocarcinoma.
†Includes all other primary lung carcinomas reclassified histologically for this study.

APPENDIX C.—Distribution by histologic types of lung carcinomas observed at necropsy for men according to time interval of death—B criteria

| Histologic type | All ages | | | | | | All men Ages 45–64 | | | | | | Ages 65+ | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1927–34 | 1935–39 | 1940–44 | 1945–49 | 1950–54 | 1955–57 | 1927–34 | 1935–39 | 1940–44 | 1945–49 | 1950–54 | 1955–57 | 1927–34 | 1935–39 | 1940–44 | 1945–49 | 1950–55 | 1955–57 |
| Adenocarcinoma | 4 | 21 | 36 | 38 | 58 | 53 | 2 | 15 | 24 | 24 | 23 | 25 | 2 | 6 | 11 | 12 | 27 | 24 |
| Differentiated | 1 | 8 | 12 | 20 | 30 | 17 | 1 | 6 | 4 | 12 | 13 | 8 | — | 2 | 7 | 6 | 12 | 9 |
| Cytocohesive | 3 | 6 | 16 | 6 | 5 | 14 | 1 | 4 | 12 | 2 | 1 | 4 | 2 | 2 | 4 | 4 | 3 | 7 |
| Giant and spindle-cell | — | 7 | 8 | 12 | 23 | 22 | — | 5 | 8 | 10 | 9 | 13 | — | 2 | — | 2 | 12 | 8 |
| Epidermoid | 15 | 25 | 51 | 55 | 64 | 45 | 11 | 20 | 30 | 34 | 29 | 23 | 4 | 4 | 16 | 17 | 34 | 22 |
| Anaplastic | 14 | 31 | 39 | 51 | 51 | 38 | 10 | 18 | 22 | 26 | 27 | 23 | 1 | 9 | 11 | 21 | 23 | 15 |
| Mixed* | 1 | 3 | 8 | 6 | 8 | 4 | 1 | 2 | 5 | 5 | 4 | 1 | — | — | 3 | 1 | 3 | 3 |
| Bronchiolar | — | 1 | 4 | 4 | 7 | 2 | — | — | 3 | 2 | 2 | — | — | — | 1 | 2 | 5 | 2 |
| Other† | 1 | — | 4 | 4 | 8 | 5 | — | — | 1 | 2 | 3 | 3 | — | — | 2 | 2 | 3 | 1 |
| Total | 35 | 81 | 142 | 158 | 196 | 147 | 24 | 55 | 85 | 93 | 88 | 75 | 7 | 19 | 44 | 55 | 95 | 67 |
| | | | | | | | Caucasian men | | | | | | | | | | | |
| Adenocarcinoma | 4 | 17 | 30 | 37 | 47 | 42 | 2 | 12 | 19 | 23 | 20 | 19 | 2 | 5 | 10 | 12 | 21 | 20 |
| Differentiated | 1 | 5 | 10 | 19 | 22 | 13 | 1 | 4 | 3 | 11 | 11 | 6 | — | 1 | 6 | 6 | 8 | 7 |
| Cytocohesive | 3 | 6 | 12 | 6 | 5 | 13 | 1 | 4 | 8 | 2 | 1 | 4 | 2 | 2 | 4 | 4 | 3 | 7 |
| Giant and spindle-cell | — | 6 | 8 | 12 | 20 | 16 | — | 4 | 8 | 10 | 8 | 9 | — | 2 | — | 2 | 10 | 6 |
| Epidermoid | 12 | 23 | 43 | 45 | 53 | 38 | 9 | 18 | 23 | 28 | 21 | 20 | 3 | 4 | 15 | 15 | 32 | 18 |
| Anaplastic | 7 | 29 | 33 | 41 | 47 | 33 | 6 | 16 | 18 | 23 | 25 | 19 | — | 9 | 10 | 16 | 21 | 14 |
| Mixed* | 1 | 2 | 5 | 4 | 6 | 3 | 1 | 1 | 4 | 3 | 3 | 1 | — | — | 1 | 1 | 2 | 2 |
| Bronchiolar | — | 1 | 3 | 3 | 7 | 1 | — | — | 2 | 2 | 2 | — | — | — | 1 | 1 | 5 | 1 |
| Other† | 1 | — | 4 | 4 | 5 | 5 | — | — | 1 | 2 | 3 | 3 | — | — | 2 | 2 | 2 | 1 |
| Total | 25 | 72 | 118 | 134 | 165 | 122 | 18 | 47 | 67 | 81 | 74 | 62 | 5 | 18 | 39 | 47 | 83 | 56 |

*Combined epidermoid and adenocarcinoma.
†Includes all other primary lung carcinomas reclassified histologically for this study (bronchial adenomas and unclassified carcinomas).

APPENDIX D.—Distribution by histologic types of lung carcinomas observed at necropsy for women according to time interval of death—B criteria

| | All women | | | | | |
|---|---|---|---|---|---|---|
| | All ages | | Ages 45–64 | | Ages 65+ | |
| Histologic type | 1927–44 | 1945–57 | 1927–44 | 1945–57 | 1927–44 | 1945–57 |
| Adenocarcinoma | 18 | 49 | 10 | 16 | 5 | 27 |
| Differentiated | 5 | 26 | 3 | 8 | — | 14 |
| Cytocohesive | 10 | 8 | 6 | 1 | 3 | 6 |
| Giant and spindle-cell | 3 | 15 | 1 | 7 | 2 | 7 |
| Epidermoid | 10 | 17 | 6 | 7 | 2 | 7 |
| Anaplastic | 10 | 17 | 7 | 10 | 1 | 7 |
| Mixed* | 4 | 1 | — | 1 | 4 | — |
| Bronchiolar | 9 | 3 | 3 | 1 | 6 | 2 |
| Other† | 1 | 4 | — | 1 | 1 | 3 |
| Total | 52 | 91 | 26 | 36 | 19 | 46 |
| | Caucasian women | | | | | |
| Adenocarcinoma | 13 | 31 | 8 | 11 | 4 | 16 |
| Differentiated | 4 | 17 | 3 | 5 | — | 10 |
| Cytocohesive | 7 | 2 | 5 | — | 2 | 1 |
| Giant and spindle-cell | 2 | 12 | — | 6 | 2 | 5 |
| Epidermoid | 4 | 9 | 3 | 4 | 1 | 3 |
| Anaplastic | 9 | 10 | 6 | 5 | 1 | 5 |
| Mixed* | 4 | — | — | — | 4 | — |
| Bronchiolar | 7 | 1 | 1 | 1 | 6 | — |
| Other† | — | 1 | — | — | — | 1 |
| Total | 31 | 52 | 18 | 21 | 16 | 25 |

* Combined epidermoid and adenocarcinoma.

† Includes all other primary lung carcinomas reclassified histologically for this study (bronchiolar carcinomas, bronchial adenomas, and unclassified carcinomas).

APPENDIX E.—Distribution by histologic types of lung carcinomas observed at necropsy in Mexicans according to time interval of death—B criteria

| | 1927–34 | 1935–39 | 1940–44 | 1945–49 | 1950–54 | 1955–57 |
|---|---|---|---|---|---|---|
| | | | Men | | | |
| All primary lung carcinomas | 6 | 7 | 20 | 12 | 15 | 9 |
| Total number of lung carcinomas reclassified histologically for this study | 6 | 7 | 17 | 12 | 15 | 9 |
| Adenocarcinoma | — | 3 | 5 | — | 4 | 4 |
| Differentiated | — | 3 | 2 | — | 3 | 1 |
| Cytocohesive | — | — | 3 | — | — | — |
| Giant and spindle-cell | — | — | — | — | 1 | 3 |
| Epidermoid | 2 | 1 | 5 | 5 | 6 | 2 |
| Anaplastic | 4 | 2 | 6 | 6 | 2 | 1 |
| Mixed* | — | 1 | 1 | 1 | 1 | 1 |
| Bronchiolar | — | — | — | — | — | 1 |
| Other† | — | — | — | — | 2 | — |
| | | | Women | | | |
| All primary lung carcinomas | 5 | 5 | 6 | 9 | 8 | 13 |
| Total number of lung carcinomas reclassified histologically for this study | 5 | 4 | 5 | 9 | 8 | 13 |
| Adenocarcinoma | 2 | 1 | 1 | 5 | 1 | 8 |
| Differentiated | 1 | — | — | 4 | — | 5 |
| Cytocohesive | 1 | — | 1 | — | — | 2 |
| Giant and spindle-cell | — | 1 | — | 1 | 1 | 1 |
| Epidermoid | 2 | 2 | 2 | 1 | 5 | 1 |
| Anaplastic | 1 | — | — | 1 | 1 | 3 |
| Mixed* | — | — | — | 1 | — | — |
| Bronchiolar | — | 1 | 1 | 1 | — | — |
| Other† | — | — | 1 | — | 1 | 1 |

* Combined epidermoid and adenocarcinoma.

† Includes all other primary lung carcinomas reclassified histologically for this study (bronchial adenomas and unclassified carcinomas).

APPENDIX F.—Distribution of lung carcinomas observed at necropsy by histologic types, sex and time. Data are compared with those of O'Neal *et al.*, and Spain to show differences—A criteria

## Men

| Present study | | | | O'Neal *et al.* (*10*) | | | | | Spain (*11*) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1) Epidermoid and undifferentiated | 2) Adenocarcinoma* | 3) Mixed† | | 1) Epidermoid and undifferentiated | | 2) Adenocarcinoma* | | | 1) Epidermoid and undifferentiated | | 2) Adenocarcinoma* | |
| | | | | | Necropsies‡ | | | | | Necropsies‡ | | | |
| Year | Number | Number | Number | Year | Number | Percent | Number | Percent | Year | Number | Percent | Number | Percent |
| — | — | — | — | 1910–19 | 3 | (1.1) | 0 | — | 1912–21 | 2 | (0.4) | 3 | (0.5) |
| 1927–34 | 30 | 1 | 2 | 1920–29 | 2 | (0.3) | 1 | (0.2) | 1922–31 | 12 | (1.4) | 4 | (0.5) |
| 1935–39 | 67 | 6 | 6 | 1930–39 | 39 | (3.4) | 10 | (0.9) | 1932–41 | 50 | (3.2) | 19 | (1.2) |
| 1940–44 | 103 | 9 | 15 | 1940–44 | 32 | (3.7) | 5 | (0.6) | 1942–46 | 31 | (4.0) | 9 | (1.2) |
| 1945–49 | 109 | 22 | 6 | 1945–54 | 139 | (6.5) | 25 | (1.2) | 1947–56 | 75 | (4.4) | 20 | (1.2) |
| 1950–54 | 120 | 25 | 7 | — | — | — | — | — | — | — | — | — | — |
| 1955–57 | 96 | 14 | 9 | — | — | — | — | — | — | — | — | — | — |
| Total | 525 | 77 | 45 | | 215 | (4.2) | 41 | (0.8) | | 170 | (3.1) | 55 | (1.0) |

## Women§

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| — | — | — | — | 1910–19 | 0 | (0 ) | 0 | (0 ) | 1912–21 | 0 | (0 ) | 1 | (0.2) |
| 1927–34 | 7 | 2 | 1 | 1920–29 | 1 | (0.2) | 4 | (1.0) | 1922–31 | 0 | (0 ) | 5 | (0.6) |
| 1935–39 | 6 | 4 | 5 | 1930–39 | 4 | (0.6) | 4 | (0.6) | 1932–41 | 5 | (0.4) | 9 | (0.6) |
| 1940–44 | 14 | 0 | 3 | 1940–44 | 6 | (1.3) | 1 | (0.2) | 1942–46 | 4 | (0.6) | 5 | (0.8) |
| 1945–49 | 9 | 4 | 1 | 1945–54 | 13 | (1.0) | 12 | (0.9) | 1947–56 | 15 | (1.0) | 10 | (0.7) |
| 1950–54 | 16 | 7 | 1 | — | — | — | — | — | — | — | — | — | — |
| 1955–57 | 18 | 8 | 2 | — | — | — | — | — | — | — | — | — | — |
| Total | 70 | 25 | 13 | | 24 | (0.8) | 21 | (0.7) | | 24 | (0.6) | 30 | (0.6) |

*Excludes bronchiolar and mixed tumors.
†Mixed epidermoid and adenocarcinoma, only. Excludes bronchiolar tumors.
‡Adult men.
§Adult women.

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# PLATES

## PLATE 171

FIGURE 1.—Photomicrograph of a field from primary pulmonary neoplasm located in the right primary bronchus. Neoplasm was designated as anaplastic giant- and spindle-cell carcinoma by use of A criteria, and as giant-cell adenocarcinoma by use of B criteria. Intracytoplasmic mucopolysaccharide is resistant to diastase digestion. Periodic acid-Schiff after diastase digestion. × 500

FIGURE 2.—Photomicrograph of primary pulmonary neoplasm located in left upper lobe bronchus. Neoplasm was classified as giant-cell epidermoid carcinoma by use of A criteria and giant-cell adenocarcinoma by use of B criteria. Intracytoplasmic acid mucopolysaccharide stains blue. Alcian blue-orange G. × 500

FIGURE 3.—High magnification of a field from primary tumor located in segmental bronchus, left upper lobe. Neoplasm was designated as epidermoid carcinoma by A criteria and adenocarcinoma, cytocohesive variant, by B criteria. Intracytoplasmic acid mucopolysaccharide within cytocohesive nests of tumor cells stains blue-green. Alcian blue-saffron. × 500

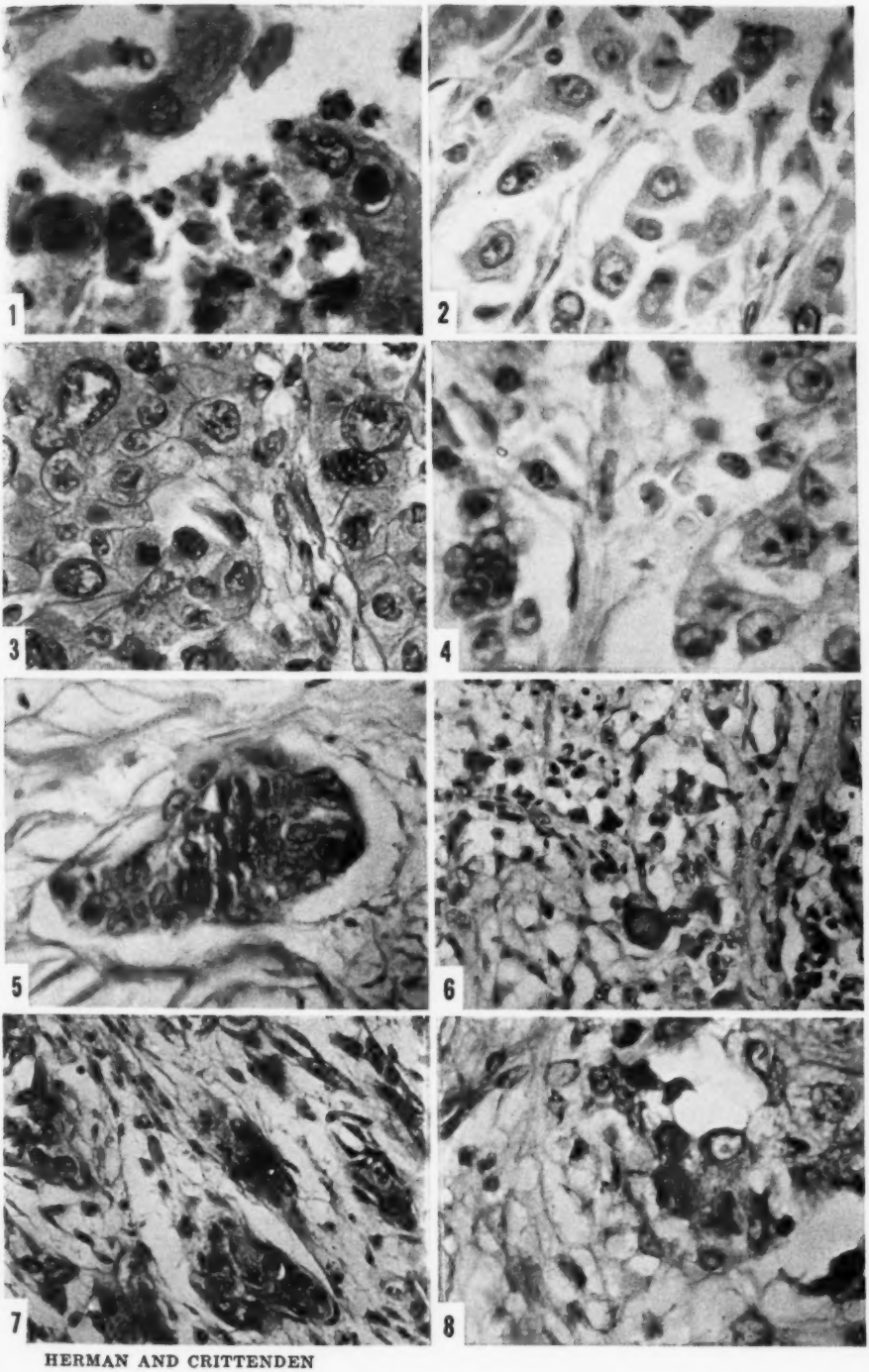
FIGURE 4.—Photomicrograph of a field of undifferentiated primary lung carcinoma. Neoplasm was designated as large cell anaplastic carcinoma by A criteria and adenocarcinoma, grade IV, by B criteria. Large nucleoli, as demonstrated in this neoplasm, was a feature frequently observed in poorly differentiated adenocarcinoma. Alcian blue-orange G. × 500

FIGURE 5.—Photomicrograph of field from primary pulmonary neoplasm showing intracytoplasmic acid mucopolysaccharide in cytocohesive nests. Tumor was composed of definite acini and solid nests and was classified as mixed epidermoid and adenocarcinoma by A criteria, and adenocarcinoma by B criteria. Alcian blue-saffron. × 275

FIGURE 6.—Photomicrograph of primary pulmonary neoplasm located in primary bronchus showing a field of undifferentiated carcinoma with giant-cell formation. Tumor was classified as epidermoid carcinoma according to both A and B criteria. Keratin stains red with saffron and erythosin. Alcian blue-saffron. × 250

FIGURE 7.—Photomicrograph of a field from peripheral pulmonary tumor, to demonstrate keratin formation. Neoplasm was designated as epidermoid carcinoma according to A criteria and mixed epidermoid and adenocarcinoma according to B criteria. Alcian blue-saffron. × 250

FIGURE 8.—Higher magnification of another field of neoplasm illustrated in figure 7 to demonstrate intracytoplasmic acid mucopolysaccharide. Alcian blue-saffron. × 500

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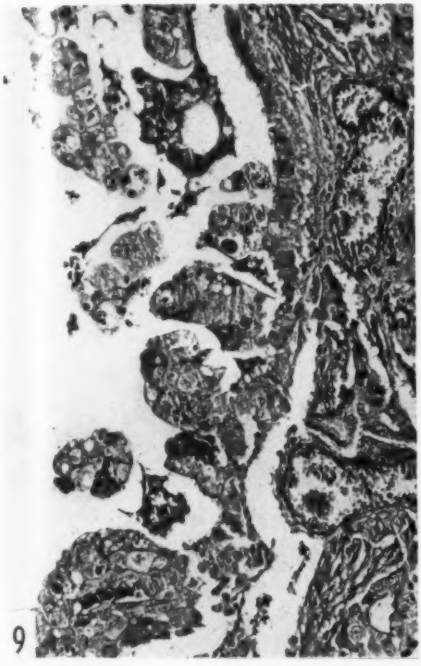
## PLATE 172

FIGURE 9.—Photomicrograph of another field of the same tumor illustrated in figure 3. The surface epithelium shows neoplastic changes and tumor cells contain PAS-positive, diastase-resistant material. Periodic acid-Schiff reaction after diastase digestion. × 125

FIGURE 10.—Photomicrograph of another field of same neoplasm to demonstrate cytocohesive nests of tumor cells that simulate epidermoid carcinoma. Tumor was classified as epidermoid carcinoma by "A" criteria and adenocarcinoma, cytocohesive variant, by "B" criteria. Hematoxylin and eosin. × 75

FIGURE 11.—Another area of tumor, in figures 3, 9, and 10, showing an acinar structure within a lymphatic. Hematoxylin and eosin. × 125

FIGURE 12.—Additional field of same tumor illustrating reactive fibrosis surrounding poorly formed acini and tubular structures. Hematoxylin and eosin. × 125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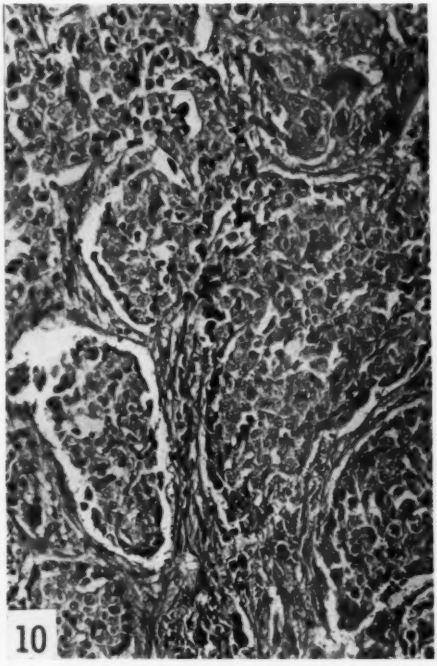
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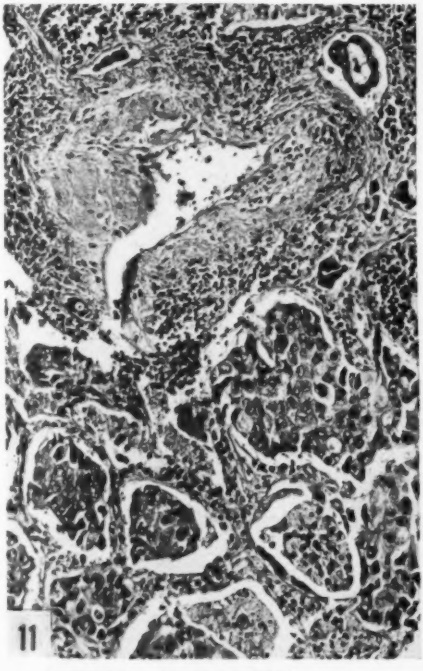
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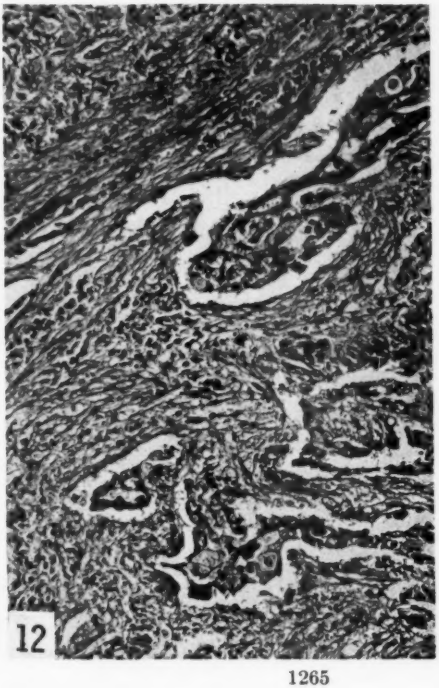
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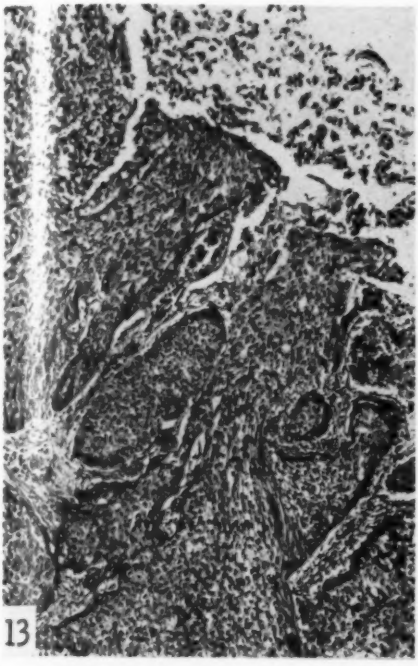
PLATE 173

FIGURE 13.—Photomicrograph of another field of same tumor as illustrated in figure 6, demonstrating progression from well-differentiated to undifferentiated carcinoma with giant-cell formation. Tumor was classified as epidermoid carcinoma according to both A and B criteria. Keratin stains red with saffron. Alcian blue-saffron. × 75

FIGURE 14.—Low magnification of same tumor as shown in figure 2, demonstrating pattern of growth around large bronchus. Neoplasm was designated as giant-cell adenocarcinoma by use of B criteria. Periodic acid-Schiff reaction after diastase digestion. × 75

FIGURE 15.—Field of tumor located in primary bronchus showing both acinar pattern and solid nests. Neoplasm was classified as mixed epidermoid and adenocarcinoma by A criteria and adenocarcinoma by B criteria. Hematoxylin and eosin. × 125

FIGURE 16.—Peripheral area of same neoplasm as shown in figure 15, illustrating papillary growth that simulates bronchiolar carcinoma. Hematoxylin and eosin. × 125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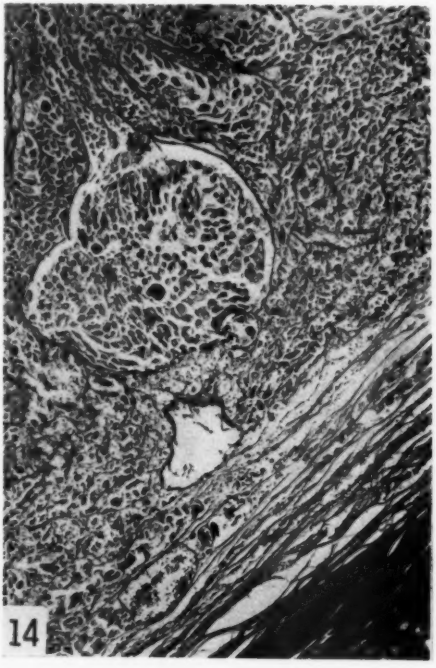
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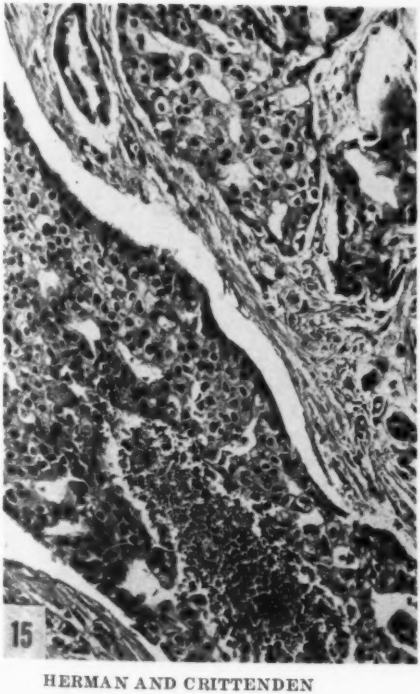
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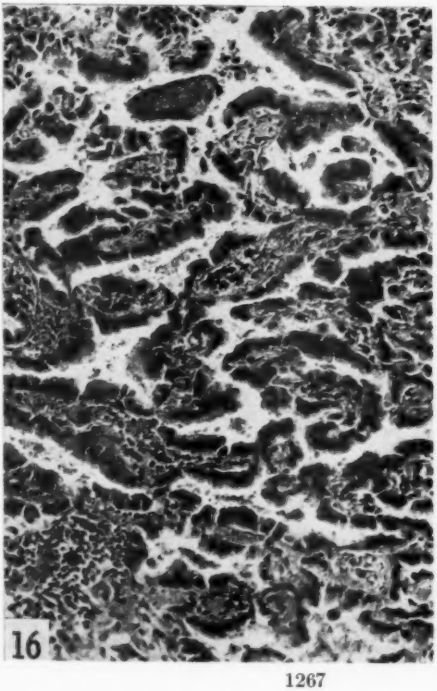
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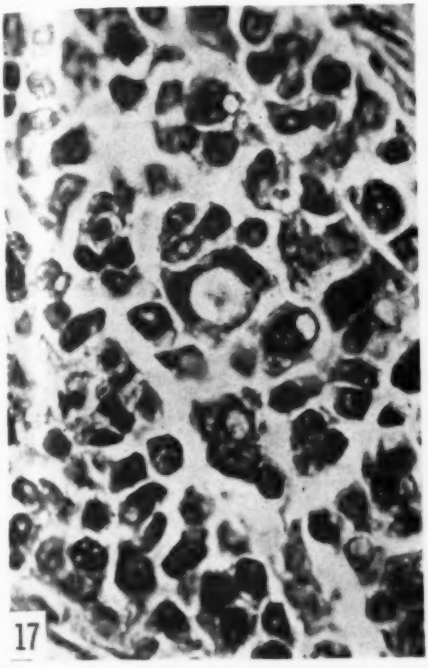
PLATE 174

FIGURE 17.—High magnification of representative field of lung tumor showing signet-cell formation. Intracytoplasmic acid mucopolysaccharide was demonstrated by use of Alcian blue. Neoplasm was designated as large-cell anaplastic carcinoma by A criteria and giant-cell adenocarcinoma by B criteria. Hematoxylin and eosin. × 500

FIGURE 18.—Low-power field illustrating pattern of tumor growth around right primary bronchus. Neoplasm was designated as anaplastic giant-cell carcinoma by A criteria, and giant-cell adenocarcinoma by B criteria. Hematoxylin and eosin. × 55

FIGURE 19.—Photomicrograph of field from carcinoma located in the right middle lobe bronchus. Neoplasm was classified as giant-cell epidermoid carcinoma by A criteria and giant-cell adenocarcinoma by B criteria. Leukophagocytosis demonstrated here was observed frequently, but was not a constant feature of this type of neoplasm. Hematoxylin and eosin. × 800

FIGURE 20.—High-power photomicrograph of field from primary pulmonary neoplasm in the right upper lobe bronchus to demonstrate intracytoplasmic acid mucopolysaccharide and anthracotic pigment within tumor cells. Tumor was designated as anaplastic giant- and spindle-cell carcinoma by use of A criteria, and as giant-cell adenocarcinoma by use of B criteria. Alcian blue-orange G. × 500



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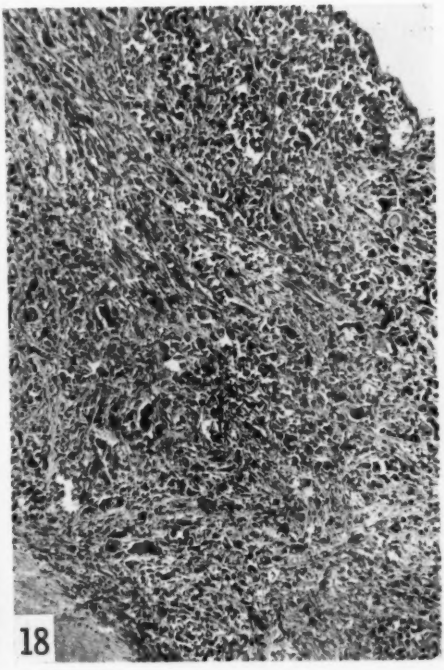

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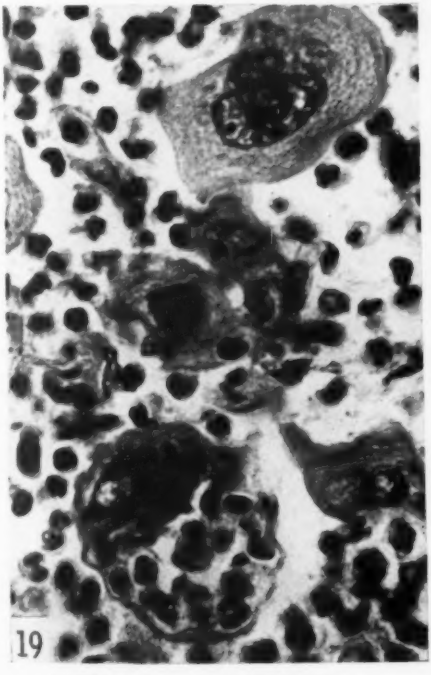

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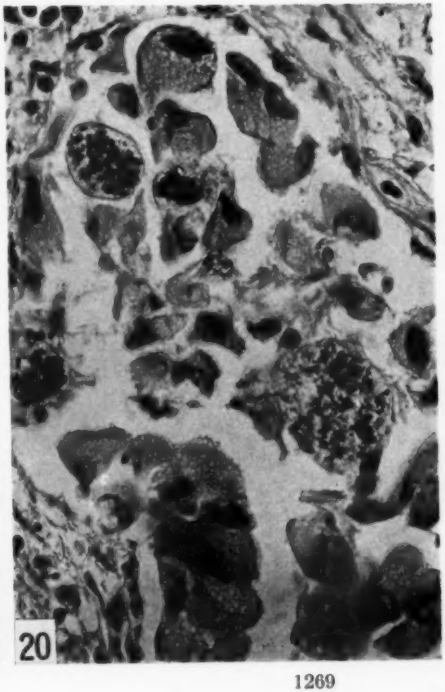

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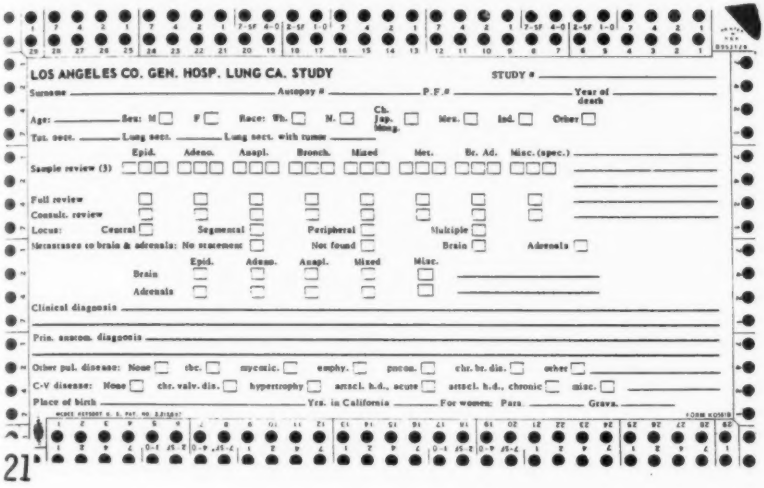
FIGURE 21.—Marginal punch card used for recording data.

FIGURE 22.—Representative field from tumor located in a right upper lobe bronchus, illustrating large tumor giant cells. Tumor was designated as anaplastic giant-cell carcinoma by A criteria and giant-cell adenocarcinoma by B criteria. Hematoxylin and eosin. × 125

FIGURE 23.—Photomicrograph of neoplasm located in primary bronchus, demonstrating nuclear debris in connective tissue. Tumor was designated as anaplastic small-cell carcinoma by both A and B criteria. Hematoxylin and eosin. × 250


LOS ANGELES CO. GEN. HOSP. LUNG CA. STUDY

STUDY #

Surname Autopsy # P.F.# Year of death

Age: Sex: M F Race: Wh. N. Ch. Jap. Negro Mex. Ind. Other

Tot. secs. Lung secs. Lung secs. with tumor

Epid. Adeno. Anapl. Bronch. Mixed Mec. Br. Ad. Misc. (spec.)

Sample review (3)

Full review

Consult. review

Locus: Central Segmental Peripheral Multiple

Metastases to brain & adrenals: No statement Not found Brain Adrenals

Epid. Adeno. Anapl. Mixed Misc.

Brain

Adrenals

Clinical diagnosis

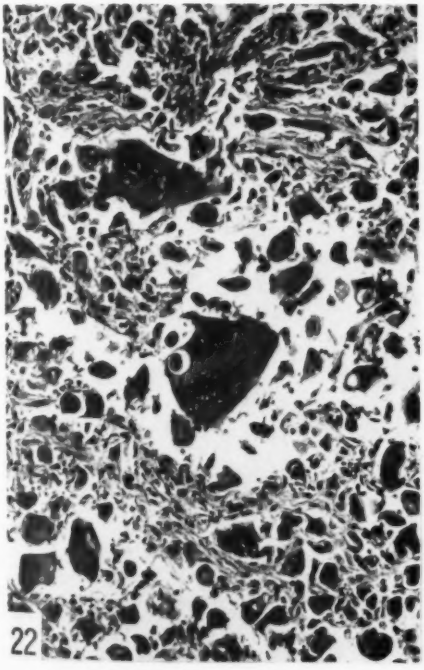
Prin. anatom. diagnosis

Other pul. disease: None tbc. mycotic. emphy. pneum. chr. br. dis. other

C-V disease: None chr. valv. dis. hypertrophy arterscl. h.d., acute arterscl. h.d., chronic misc.

Place of birth Yrs. in California For women: Para Gravs.

21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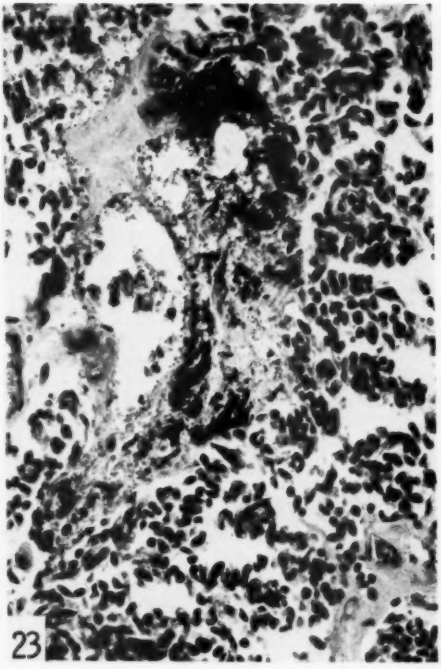
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# Induction of Mammary Cancer in Agent-Free Mice Bearing Pituitary Isografts Correlated With Inherited Hormonal Mechanisms [1, 2]

JOHN J. BITTNER *and* HARVEY L. COLE, *Division of Cancer Biology, Department of Pathology, University of Minnesota Medical School, Minneapolis, Minnesota*

SUMMARY

**Mammary cancer was induced by the subcutaneous implantation of pituitary isografts in agent-free females of strains C3Hf and BALB/c and $F_1$ hybrids of several crosses. Some ovariectomized females became cancerous when they were the recipients of pituitary isografts, as did some castrated males implanted with pituitaries alone or ovaries and pituitaries. Some mammary tumors contained a milklike secretion, several were diagnosed as adenoacanthomas or adenomas, and adrenocortical alterations were seen in gonadectomized animals bearing isografts, which included ovaries. Only (Af × C57BL/4)$F_1$ hybrids as a group did not develop mammary cancer when subjected to these different hormonal influences. An association was seen between the induction of mammary cancer in agent-free mice with hypophyseal isografts and the presence of an inherited hormonal pattern that would produce a high incidence of tumors in virgin females having other inciting factors (mammary-tumor agent and susceptibility). The development of adrenocortical lesions in gonadectomized animals of some groups when they received pituitary and/or ovarian grafts might suggest some abnormal hormonal activity and/or function.—J. Nat. Cancer Inst. 27: 1273–1284, 1961.**

SPONTANEOUS MAMMARY cancer usually develops in mice that have the mammary-tumor agent (MTA), the susceptibility to the disease, and an adequate hormonal stimulation (*1–4*, reviewed in *5*). However, the incidence may be as high as 70 percent in agent-free females of one inbred strain and their hybrids (*6–8*).

In 1939 it was found that the incidence of mammary cancer could be increased by the subcutaneous implantation of hypophyses (*9–11*), but only in females of susceptible strains that had the MTA. That the disease could be induced in agent-free females of certain inbred strains and their $F_1$ hybrids by pituitary isografts was shown by Mühlbock and Boot (*12–15*). The number of experimental females was limited by the supply of donors, for some recipients received as many as 200 isografts.

[1] Received for publication April 7, 1961; revised August 7, 1961.

[2] This investigation was supported by research grant C-372C12 from the National Cancer Institute, National Institutes of Health, Public Health Service; grant E-3 from the American Cancer Society, Inc.; and by the Elsa U. Pardee Foundation.



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These observations have been corroborated (*16–19*), and the disease may be induced by a single pituitary isograft (*18* and Mühlbock, personal communication). The presence of the MTA, the number of implants (*13, 17*), and the site of implantation (*13, 14*) influenced not only the incidence of tumors but also the average cancer age.

Various theories have been suggested to account for the development of mammary tumors after the implantation of hypophyses (*12–19*). Woolley *et al.* (*20, 21*) demonstrated that mammary cancer would develop in mice of some cancerous strains that had been gonadectomized at birth. In these castrated animals, the adrenals became hyperplastic or even cancerous, provided the hormones that stimulated the secondary sex organs, and eventually caused the development of cancer. From data observed in these investigations, various inherited hormonal mechanisms have been described that may either induce or inhibit mammary cancer in females possessing the other causative factors (reviewed in *5*). The physiological effects of these hormonal patterns were due to certain differences in activity or function inherent in the tissues themselves, as well as differences in pituitary stimuli, also probably transmitted (*22–24*).

The studies to be reported are concerned with the development of mammary cancer in mice of inbred strains and $F_1$ hybrids bearing grafts of endocrine organs correlated with the presence of inherited hormonal patterns. With the exception of some animals of strain BALB/c, all were free of the mammary-tumor agent. Certain aspects of the problem cannot be considered extensively until other groups of strain BALB/c mice, now under observation and approximately 15 months of age, have lived their lifespan.

## MATERIALS AND METHODS

Agent-free mice of strains Ax, Zb,[3] I, C57BL/4, and BALB/c and their $F_1$ hybrids were used in the experiments to be described. Another group of $F_1$ hybrids was produced by strain BALB/c females mated with males of strain C3H/AnBi. With the exception of the latter hybrids (*25*), low incidences of mammary tumors were seen (*5, 8*). Another group of strain BALB/c females possessed the MTA, obtained either by the administration of tumor extracts or foster nursing. Other details concerning the history of the various strains, as well as cancer data, will be found in recent publications (*5, 8*).

The pituitary isografts were placed subcutaneously in the right-axillary region of the recipients by trocar, usually in pools of 3 or 5. The donors ranged from 3 to 8 weeks of age. When we planned the protocols for these experiments, groups of recipients were to have been implanted with isografts from donors of one sex, but the scarcity of donors at certain

[3] Ed. note: Since Dr. Bittner does not use the nomenclature as recommended by the Committee on Standardized Genetic Nomenclature for Mice (Cancer Res. 20: 145–169, 1960), the following explanations are given: Ax = Af; Z = strain C3H; Zb = C3Hf.

periods, due to death from infantile diarrhea, made this impossible. Details are given for each group of experimental animals. Females were maintained as nonbreeders, and all animals retained their hypophyses.

With few exceptions, the diagnosis of the mammary tumors was confirmed by histological examination. Since many mice are still living, detailed information cannot be given about adrenal alterations in all experimental animals.

Several mammary tumors were tested by biological assay for the MTA, as described previously (*8*).

## EXPERIMENTAL RESULTS

The incidence of mammary cancer in agent-free breeding females of strain BALB/c was less than 1 percent, whereas when they possessed the MTA, the incidence increased to over 70 percent with an early average cancer age (*25* and table 1). Data are presented in table 1 on the appearance of mammary cancer in strain BALB/c mice with the MTA when they were subjected to various hormonal influences, which included the implantation subcutaneously of isografts of pituitaries from donors of strain BALB/c. Strain BALB/c females that received pituitary isografts between the ages of 4 and 14 days developed mammary tumors as early as did breeders, though the recipients were maintained as nonbreeders. Ovariectomized strain BALB/c females with the MTA had mammary tumors after the subcutaneous implantation of hypophyses, and in these females there was an indication that male pituitaries were more effective than grafts from female donors.

TABLE 1.—Development of mammary cancer in mice of strain BALB/c that possess the MTA under different experimental conditions, including isografts of hypophyses

| Group | Experimental protocol | Number | Incidence of cancer (percent) | Average age in days | |
|---|---|---|---|---|---|
| | | | | Cancer | Noncancerous |
| 1 | Females as normal breeders | 119 | 71 | 284 | 459 |
| 2 | Females, 3 pituitaries between 4–14 days of age | 19 | 63 | 273 | 408 |
| 3 | Ovariectomized at 4 weeks: 3 male pituitaries at 3 months | 7 | 86 | 319 | 371 |
| | Ovariectomized at 4 weeks: 3 female pituitaries at 3 months | 6 | 50 | 359 | 347 |
| 4 | Ovariectomized at 4 weeks, 3 pituitaries and 2 adrenals implanted between 7.5 and 8 months | 11 | 64 | 440 | 449 |
| 5 | Intact females, starting at 8 weeks, 3 ovaries grafted, right axilla, total: 24 | 8 | 50 | 362 | 804 |
| 6 | Males, castrated at 5 weeks, 3 ovaries implanted at 7–10 weeks, total: 24 at intervals of 1–6 weeks | 19 | 74 | 385 | 525 |

Castrated strain BALB/c males bearing ovarian grafts had approximately the same incidence of mammary cancer as did breeders, though the tumors appeared later in the males. One mammary tumor from an experimental male was tested for the MTA (table 5, expt. 756). The mean incidence in the test animals was 64 percent, with an average cancer age of 398 days. This demonstrated the tumor-inducing activity of the MTA.

The observations for agent-free animals of strain BALB/c are presented in table 2. Both ovaries and one adrenal were removed from 6-week-old females, the only group remaining noncancerous (group 2). Starting at 4 weeks of age, four strain BALB/c females were implanted with pituitaries, and, after receiving an average of 36 grafts, all developed mammary cancer at an average age of 334 days. Others were implanted with only 5 pituitaries at 2.5 months and had a low incidence at an extremely late age (group 3). Tumors were observed in females of 2 groups that were ovariectomized and implanted with hypophyses, and in which the incidence and age were influenced by the number of isografts.

Five agent-free males of strain BALB/c were castrated when 4 weeks of age and were the recipients of both ovaries and pituitaries, as given in the protocol of group 6 (table 2). Four of these males had mammary cancer at an average age of 582 days.

An adenocarcinoma from a cancerous female of group 5 (table 2) was tested for the MTA (table 5, expt. 758). The low incidence and the late cancer age observed in the assay animals showed that the tumor did not contain the MTA. The donor of the assayed mammary tumor had adrenals with cortical lesions.

One ovariectomized strain BALB/c female (group 5, table 2) had an adenoacanthoma when 415 days old. There were adrenocortical lesions among females of group 2 and a male that had been castrated and was

TABLE 2.—Development of mammary cancer in agent-free mice of strain BALB/c under different experimental conditions, which included isografts of hypophyses

| Group | Experimental protocol | Number | Incidence of cancer (percent) | Average age in days | |
|---|---|---|---|---|---|
| | | | | Cancer | Noncancerous |
| 1 | Intact females, pituitaries implanted starting at 4 weeks, total: 15–40; average: 36 | 4 | 100 | 334 | — |
| 2 | Females bilaterally ovariectomized and unilaterally adrenalectomized at 6 weeks | 13 | 0 | — | 680 |
| 3 | Intact females, 5 pituitaries implanted at 2.5 months | 4 | 25 | 574 | 800 |
| 4 | Females ovariectomized at 4 weeks, 5 pituitaries implanted at 2.5 months | 9 | 44 | 826 | 774 |
| 5 | Females ovariectomized at 4 weeks, pituitaries implanted starting at 3.0 months, total: 30 | 5 | 100 | 394 | — |
| 6 | Males, castrated at 4 weeks, 3 ovaries grafted between 4–6 weeks, 3 pituitaries implanted at 3 months | 5 | 80 | 582 | 403 |

the recipient of both ovarian and pituitary isografts. Several of the pituitary-induced mammary tumors contained a milklike secretion.

Table 3 shows data for females of strain Zb and certain $F_1$ hybrids that were all devoid of the MTA.

Eight intact strain Zb females received an average of 40 pituitary isografts and 4 developed mammary cancer, while 8-week-old ovariectomized females remained free of the disease after being implanted with a smaller number of hypophyses. Females of this latter group survived to an average age of only 513 days. However, mammary tumors were observed among ovariectomized $F_1$ females of the Zb $\times$ I and BALB/c $\times$ C3H/AnBi crosses, with the incidence and age determined by age at ovariectomy and number of isografts. One castrated male of the latter group had mammary cancer when 711 days old.

Two mammary tumors from strain Zb females were assayed, and the data failed to demonstrate the MTA in the tumors (table 5).

Mammary tumors developed in a higher incidence and at an earlier cancer age in (Zb $\times$ I)$F_1$ females that had been ovariectomized between 6 and 8 weeks of age when they were implanted with 30 $F_1$ hypophyseal isografts than when only 6 grafts were used. Four additional hybrids of the latter group died before they were 1 year old and so have not been included. Tumors failed to develop in (Zb $\times$ I)$F_1$ castrated males that received only pituitary implants.

In the hybrids produced by mating strain BALB/c females with males of strain C3H/AnBi, mammary tumors developed in 69 percent of the 13 $F_1$ females that had been ovariectomized at 4 weeks of age and given from 6 to 20 $F_1$ pituitaries. There were cortical changes in the adrenals from several of these experimental females. One castrated $F_1$ male with pituitary implants had mammary cancer at the extreme old age of 711 days.

Table 4 lists the hybrids having maternal parents of the agent-free strain Ax. Mammary tumors failed to develop in 30 intact or ovariectomized (Ax $\times$ Zb)$F_1$ females, though these noncancerous females survived to an average age of approximately 24 months. Three intact $F_1$ females were implanted subcutaneously with an average of 50 $F_1$ pituitaries, and 2 had tumors at an average age of 689 days; one tumor was diagnosed as an adenoacanthoma. Twenty-five other (Ax $\times$ Zb)$F_1$ females were ovariectomized at 6 to 8 weeks of age, and each received from 30 to 55 pituitary isografts, mostly from $F_1$ female donors. Six mammary tumors were found in the mice at an average age of over 20 months; one tumor was an adenoacanthoma and another was an adenoma of mammary origin.

Thirty-two (Ax $\times$ Zb)$F_1$ males were castrated at 4 weeks of age. Those that did not receive pituitary implants remained noncancerous, while those having isografts showed an incidence of 28 percent. The cancerous males had an average of 33 grafts, while 45 pituitaries were the average number implanted into males that failed to develop tumors. One of the adenocarcinomas had lung metastases.

TABLE 3.—Observations on the development of mammary cancer in agent-free mice implanted with isografts of hypophyses under different hormonal influences

| Stock | Experimental protocol | Number | Incidence of cancer (percent) | Average age in days | |
|---|---|---|---|---|---|
| | | | | Cancer | Non-cancerous |
| Zb | Intact females, female pituitaries starting at 6 weeks, total: 39–41 | 8 | 50 | 417 | 489 |
| Zb | Ovariectomized at 8 weeks, female pituitaries starting at 8 weeks, total: 9–15 | 9 | 0 | — | 513 |
| (Zb × I)$F_1$ | Ovariectomized at 6 weeks, 3 $F_1$ female pituitaries starting at 10–12 weeks, total: 30 | 5 | 80 | 279 | 468 |
| (Zb × I)$F_1$ | Ovariectomized at 8 weeks, 3 $F_1$ female pituitaries starting at 10 weeks, total: 6 | 3 | 33 | 610 | 614 |
| (Zb × I)$F_1$ | Males castrated at 6–8 weeks, total: 9–12 $F_1$ pituitaries | 12 | 0 | — | 550 |
| (BALB/c × C3H/AnBi)$F_1$ | Ovariectomized at 4 weeks, $F_1$ pituitaries starting at 4 weeks, total: 6–20 | 13 | 69 | 539 | 566 |
| (BALB/c × C3H/AnBi)$F_1$ | Males castrated at 4–5 weeks, $F_1$ pituitaries starting at 4–5 weeks, total: 6–20 | 8 | 13 | 711 | 773 |

TABLE 4.—Observations on the development of mammary cancer in agent-free mice under different hormonal influences

| Hybrids | Experimental protocol | Number | Incidence of cancer (percent) | Average age in days | |
|---|---|---|---|---|---|
| | | | | Cancer | Non-cancerous |
| (Ax × Zb)$F_1$ | Intact females, no pituitary implants | 9 | 0 | — | 732 |
| (Ax × Zb)$F_1$ | Females ovariectomized at 2 months, no implants | 21 | 0 | — | 717 |
| (Ax × Zb)$F_1$ | Intact females, ♀ $F_1$ pituitaries starting at 8 weeks, total: 46–55 | 3 | 67 | 689 | 417 |
| (Ax × Zb)$F_1$ | Females ovariectomized at 6–8 weeks, $F_1$ pituitaries mostly female, starting at 6–8 weeks, total: 30–55 | 25 | 24 | 616 | 670 |
| (Ax × Zb)$F_1$ | Males castrated at 4 weeks, no pituitary implants | 14 | 0 | — | 806 |
| (Ax × Zb)$F_1$ | Males castrated at 4–6 weeks, $F_1$♂ pituitaries starting at 5–6 weeks; + = average, 33 pituitaries; − = average, 45 pituitaries | 18 | 28 | 505 | 685 |
| (Ax × I)$F_1$ | Females ovariectomized at 4–5 weeks, $F_1$ pituitaries starting at 6 weeks, total: 24 | 5 | 20 | 520 | 607 |
| (Ax × I)$F_1$ | Males, castrated at 3 months, 3 $F_1$ ovaries and 3 $F_1$ pituitaries starting at 4 months, total pituitaries: 3–12 | 11 | 9 | 637 | 584 |
| (Ax × C57BL/4)$F_1$ | Intact females, $F_1$ pituitaries starting at 6 weeks, total: 9–12 | 8 | 0 | — | 729 |
| (Ax × C57BL/4)$F_1$ | Females, ovariectomized at 6 weeks, $F_1$ pituitaries starting at 6 weeks, total: 9–15 | 10 | 0 | — | 774 |
| (Ax × C57BL/4)$F_1$ | Males, castrated at 6 weeks, 3 $F_1$ ovaries and 3 $F_1$ pituitaries starting at 6 weeks, total pituitaries: 9–12 | 8 | 0 | — | 683 |

TABLE 5.—Biological assay of mammary tumors for the MTA

| Experiment No. | Source of mammary tumor | Grams equivalent injected | Number | Incidence of cancer (percent) | Average age in days | |
|---|---|---|---|---|---|---|
| | | | | | Cancer | Noncancerous |
| 756 | BALB/c possessing MTA, castrated at 5 weeks, ovaries implanted 10th week, total: 33. Cancer at 303 days | $2 \times 10^{-2}$ | 16 | 50 | 389 | 500 |
| | | $10^{-3}$ | 20 | 65 | 427 | 469 |
| | | $10^{-4}$ | 14 | 79 | 371 | 389 |
| 758 | BALB/c, pituitaries starting at 4 weeks, total: 35. Cancer at 386 days | $2 \times 10^{-1}$ | 15 | 13 | 495 | 588 |
| 759 | BALB/c, ovariectomized at 4 weeks, pituitaries starting at 3 months, total: 30. Cancer at 346 days | $2 \times 10^{-1}$ | 18 | 6 | 580 | 615 |
| 783 | Zb, pituitaries starting at 6 weeks, total: 39. Cancer at 411 days | $2 \times 10^{-2}$ | 20 | 5 | 557 | 548 |
| 784 | Zb, pituitaries starting at 6 weeks of age, total: 39. Cancer at 380 days | $2 \times 10^{-2}$ | 20 | 0 | — | 576 |

Eighteen females and 8 males of the (Ax × C57BL/4)$F_1$ hybrid population were observed, and all died free of mammary cancer (table 4). Included in the group were 8 intact $F_1$ females that received from 9 to 12 $F_1$ hypophyseal isografts, 10 $F_1$ females that were ovariectomized and implanted with 9 to 15 pituitary grafts, and 8 $F_1$ males that were castrated at 6 weeks of age and implanted at the same age with ovaries and pituitaries from $F_1$ donors. These $F_1$ male recipients were grafted with 9 to 12 hypophyses.

## DISCUSSION

Various theories have been suggested to explain the hormonal effects resulting from the implantation of hypophyseal isografts and the development of mammary cancer in agent-free females. Mühlbock and Boot (*12–15*) found evidence that the implants released prolactin continuously and this resulted in a double function having both mammotropic and luteotropic effects (*13*). The data obtained by Liebelt and Liebelt (*19*) would support this hypothesis, but they stated that further studies were needed to explain the hormonal mechanisms since they may be influenced by not only the MTA but by genetic factors. Haus *et al.* (*18*) observed an elevation of adrenocorticosterone in mice bearing pituitary isografts, which showed the role of the adrenals in the induction of these tumors.

Data on the development of mammary cancer in experimental animals are given in tables 1 to 4. More than 300 animals received tissue grafts, and nearly 4,300 mice served as donors. When the study was initiated in 1957, information had not been obtained concerning the number of pituitary isografts that might be needed to induce cancer, since some ani-

mals observed by Mühlbock (*13*) were the recipients of 200 implants. In our study, some mice received as many as 55 grafts, and the average number was 14 per mouse. The problem was made more difficult by our inability to obtain an adequate number of donors of some strains.

Five mammary tumors were tested for the MTA by biological assay, and the observations showed that the mammary-tumor agent could be demonstrated only in the tumor that developed in a strain BALB/c experimental male possessing the agent (table 5).

Mammary tumors were induced in agent-free females of strains BALB/c and Zb by the subcutaneous implantation of pituitary isografts as well as in (Ax × Zb)$F_1$ hybrids (tables 2, 3, and 4). Mammary cancer also was observed in agent-free females of several groups that had been ovariectomized between the ages of 4 and 8 weeks and later were the recipients of implants of hypophyses. There were a few tumors in males that had been gonadectomized and implanted with ovaries and pituitaries; another group of castrated $F_1$ hybrids had mammary cancer after they received pituitary isografts alone (table 4). We observed lung metastases in 1 male with mammary cancer.

In general, the incidences and average cancer ages were influenced by the number of pituitary isografts and the age of the recipients when they were grafted. In the gonadectomized animals, the age at the time of gonadectomy also was of considerable importance.

The only group in which mammary tumors did not develop was (Ax × C57BL/4)$F_1$ hybrids. The experimental animals included intact females with pituitary isografts, ovariectomized females with hypophyseal grafts, and $F_1$ males that had been castrated and implanted with both ovaries and pituitaries. A total of 28 mice was observed in the 3 groups.

In 1944 (*3*, *4*), it was demonstrated that the hormonal mechanism transmitted was responsible for the production of mammary cancer in virgin females of cancerous strains. Further studies showed that this inherited hormonal pattern and the inherited susceptibility to mammary cancer were not controlled by identical genes (reviewed in *5*, *26*), and mice of an inbred strain, resistant to the development of mammary cancer, transmitted the inherited hormonal pattern that would induce mammary cancer (*5*, *24*, *25*). In addition to the hormonal pattern that will induce the disease in virgin females in the presence of other causative factors, another mechanism has been described that may inhibit or delay the development of mammary cancer in breeding females (*5*).

To investigate the physiological effects of these inherited hormonal patterns, we grafted ovaries from females of parental strains into gonadectomized $F_1$ progeny (*22*). In the $F_1$ hybrids, the grafted ovaries would be subjected to the same pituitary hormonal stimulation and the hormones produced by the ovaries would be metabolized and would act upon secondary sex organs in mice with similar genetic constitutions. Evidence was obtained that certain differences of ovarian activity or function were inherent in the tissue itself, while variations in the normal histology in the ovaries of the 2 strains probably resulted from differences in the

pituitary stimuli to which they were subjected. In the hybrids, these differences disappeared when the ovaries from donors of the parental strains were subjected to stimulation from similar $F_1$ pituitaries.

By transplanting adrenals from mice of the parental strains subcutaneously into $F_1$ progeny that had been adrenalectomized-gonadectomized, we secured other data that indicated the differences observed in the postcastrational changes in adrenals of inbred strains were due to an inherited responsiveness of the adrenal tissue itself (*23*).

In other studies (*12–19*) on the induction of mammary cancer in agent-free females implanted with hypophyseal isografts, no reference was made to any hormonal characteristics inherent in the implants and/or the recipients and their possible effects upon the problem of carcinogenesis. An examination of the data revealed that tumors were induced in females having the inducing hormonal pattern, or in virgin females, with the MTA, having high incidences of mammary cancer (*5*). The purpose of this study was to determine the relationship of this inherited hormonal pattern to the induction of mammary cancer with pituitary isografts, not only in virgin females but also in gonadectomized animals in which one would expect to observe mammary cancer and also adrenocortical alterations, as demonstrated by the work of Woolley *et al.* (*20*, *21*).

In this investigation, as in previous experiments (*12–19*), mammary tumors were produced only in females of inbred strains or $F_1$ hybrids of groups in which nonbreeding females would have mammary cancer when they possessed the MTA. However, only in the (Ax × C57BL/4)$F_1$ hybrids did the animals remain noncancerous after the implantation of $F_1$ pituitaries, and the virgin females of this hybrid cross had a low incidence of mammary tumors in the presence of the agent (*2*, *5*, *27*).

Now that experimental data have been obtained for support of the theory on the association between the inducing inherited hormonal pattern and the induction of mammary cancer after the subcutaneous implantation of hypophyses, future projects will be concerned with more technical information.

In hybrid crosses between inbred strains of mice having different inherited hormonal patterns, the $F_1$ progeny would inherit characteristics from both parents. The inducing hormonal pattern for mammary cancer in mice of one parental strain would be transmitted to the $F_1$ offspring, and the hormonal activity of the endocrine glands of the progeny would correspond to the parent having the inducing hormonal pattern. This has been demonstrated by studies on the grafting of endocrine organs (*23*, *24*) and would explain the difference in response of the (Ax × Zb)$F_1$ and (Ax × C57BL/4)$F_1$ to the induction of mammary cancer. It should follow that agent-free females of our strain A, known as Ax, should not have mammary cancer after the implantation of pituitary isografts because of the low incidence of tumors in virgin females of cancerous strain A (*2*, *26*, *27*).

The mice studied in this investigation retained their own pituitaries, and yet mammary tumors did not develop until after the animals received

pituitary isografts. It is unknown whether the induction of cancer may have resulted entirely from the added stimulation of the implanted pituitary isografts or whether there might have been some synergistic effect or even some effect of the pituitary implants on the activity of the mouse's own hypophysis. The development of adrenocortical lesions in certain animals would suggest some atypical hormonal function.

A few females of the agent-free strain BALB/c were hypophysectomized before they received pituitary isografts. Although they remained noncancerous, they did not survive to extreme cancer ages as seen for some groups. Thus, this phase of the work must be repeated before any general conclusions may be made.

It was reported earlier that "Since different inherited hormonal patterns have been demonstrated in various strains of mice, further experimental studies are needed to determine the physiological relationship of these to the development of mammary cancer in breeding and virgin females and in gonadectomized animals" (*28*). To this we might add, perhaps, the induction of mammary cancer with pituitary isografts.

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# Effect of Cortisone on Skin Maintained in Organ Culture [1,2]

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SUMMARY

**Full-thickness auricular skin from adult mice was maintained in organ culture and treated with cortisone acetate for periods up to 4 weeks. The skin was floated on a complex protein medium by a technique that has been previously described. Successful grafting of the skin back to the original donor was considered a reliable criterion of the preservation of its structural integrity while in culture. Ninety-six organ cultures were maintained and subsequently grafted at weekly intervals. Cortisone acetate was added to half the cultures in an attempt to suppress the process of cell modulation in the connective-tissue layers. Gross and microscopic observations revealed that the disorganizing process occurring in the untreated cultures of skin was suppressed substantially in the cultures treated with cortisone. After 3 weeks *in vitro*, none of the control cultures could be autografted successfully, but 83 percent of the grafts treated with cortisone were transplanted successfully. It was apparent that cortisone acetate is effective in slowing the processes responsible for the degenerative modifications of organized skin *in vitro*. The effects of other steroid compounds are currently under study.—J. Nat. Cancer Inst. 27: 1285–1309, 1961.**

IN PREVIOUS publications (*1*, *2*) we described an organ-culture system for maintaining intact skin *in vitro* for several weeks. In this system, full-thickness auricular skin (epidermis, dermis, and part of the fatty layer) of adult mice is floated on the surface of a complex tissue-culture medium (*1*). After the period in culture successful grafting of the skin to the dorsum of the original donor proves that the functional ability of the skin is retained. Since auricular skin has a characteristic appearance and peculiar hair pattern, it is always distinguishable from the surrounding dorsal skin, and the course of the graft is followed easily (*3*). The skin may be treated *in vitro* with a broad spectrum of physiologically active agents incorporated into the medium or topically applied. Also, the

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effects of alterations occurring in the skin as a result of experimentation may be studied in the host animal after grafting.

We found that after 2 weeks in culture many of the skin grafts failed, though the tissue seemed viable upon gross observation. Concurrently, large numbers of embryonic cell forms were seen to proliferate on the floors of the culture flasks. Because luxuriant cell growth was observed and a number of the cultures was grafted successfully, it was assumed that the complex medium was nutritionally sufficient to maintain the cells and that the process of diffusion alone could sustain the skin. However, an obviously degenerative change was occurring in the cultured skin which ultimately altered it so that it could no longer function successfully as a graft. Preliminary histologic examination of the cultured skin revealed marked loss of cellularity and diminished collagen matrix in the dermal layers (*4*). It was then postulated that the release of the tissue from any humoral controls had caused the cells to revert to embryonic forms, which destroyed the organization of the skin and made it nonfunctional as a graft. The modulation of cells *in vitro* is an occurrence familiar to all who work with tissue cultures. If our system of organ culture was to be useful in the study of organized skin *in vitro*, a method of controlling this modulation had to be found. Since steriods, particularly those acting as anti-inflammatory agents, had proved active in suppressing and modifying fibroblasts in culture (*5–8*), we decided to test their effect on the modulation of the cells of the skin in this organ system.

## MATERIALS AND METHODS

Full-thickness auricular skin was removed from strain A white mice and placed in tissue culture by the method previously described (*1*). Skin removed from the ear served as a control, and skin from the opposite ear of the same animal was treated with cortisone in culture. In the experimental series, 0.45 mg of cortisone acetate was streaked along one side of each flask and gently flamed dry before the skin was inserted. The auricular skin was then floated on the surface of a complex protein medium (Eagle's medium with horse serum) as described earlier (*1*). All the cultures were recharged with fresh medium twice weekly. The thin film of cortisone acetate leached slowly into the medium of the experimental cultures over a 2-week period so that a fine suspension of cortisone was in intimate contact with the dermal surface. If the period of maintenance in culture exceeded 2 weeks, additional cortisone acetate was added to the experimental series.

The cortisone-treated skin grafts and the controls were maintained in culture for periods of up to 1 month. At 1-, 2-, 3-, and 4-week intervals, some of the treated cultures and their controls were removed and autografted. Some cultures were sacrificed at the same intervals and fixed in Bouin's solution for histologic examination. All cultures were photographed weekly through the flasks.

## RESULTS

Ninety-six organ cultures of auricular skin were maintained *in vitro* and subsequently autografted. Forty-eight of these cultures were treated with cortisone acetate and forty-eight served as controls. In addition, cortisone-treated grafts and controls were removed at the end of 1, 2, 3, and 4 weeks in culture and fixed for histologic procedures.

The results obtained from grafting the control and experimental series are summarized in table 1. A series done earlier, in which several protein sources in addition to horse serum were incorporated in the culture medium, is also included in the table. The additional proteins in the medium did not appreciably increase the survival of the intact skin. In contrast, both the number of successful grafts and the length of time that the skin could be maintained and then grafted were increased by the addition of cortisone acetate. Only 41 percent of the control cultures were grafted successfully after 2 weeks *in vitro*, and none retained graftability after 3 weeks in culture. Only 16 percent of the grafts on the high protein medium survived after 3 weeks in culture. Of the cultures treated with cortisone acetate, 83 percent were successfully grafted after 2 weeks, 83 percent after 3 weeks, and 16 percent survived 4 weeks *in vitro*.

TABLE 1.—Effect of cortisone acetate on the maintenance of skin grafts in organ culture

| Group No. | Culture mediums | Number of days *in vitro* | Number of autografts | Takes* Number | Takes* Percent |
|---|---|---|---|---|---|
| 1. Control | Eagle's medium plus horse serum (10%) | 7 | 12 | 10 | 83 |
| | | 14 | 12 | 5 | 41 |
| | | 21 | 12 | 0 | 0 |
| | | 28 | 12 | 0 | 0 |
| 2. Proteins added | Eagle's medium plus horse serum (10%), beef-embryo extract (5%), ascetic fluid (10%) | 21 | 12 | 2 | 16 |
| 3. Cortisone acetate added | Eagle's medium plus horse serum (10%), cortisone acetate (0.45 mg/flask) | 7 | 12 | 12 | 100 |
| | | 14 | 12 | 10 | 83 |
| | | 21 | 12 | 10 | 83 |
| | | 28 | 12 | 2 | 16 |

*Grafts which observably vascularized and retained the characteristic appearance of auricular skin after transplantation to the dorsum of the animal. Follow-up studies ranged from 3 months to 1 year.

Two pairs of organ cultures after 3 weeks *in vitro* are shown in figure 1. Initially, all the grafts were approximately the same size, *i.e.*, around 90 $mm^2$. Even in this photograph of the whole preparations the cultures treated with cortisone acetate appear to be in good condition, while the control cultures are noticeably shrunken.

### Gross Observations

After 1 week in tissue culture, some gross differences were apparent between the skin treated with cortisone and the untreated controls. The skin of the controls was shrunken and wrinkled while the treated grafts looked essentially normal (*c.f.* figs. 2, 3, 4, and 5).

At the end of 2 weeks in culture, the skin of the controls had shrunk considerably, appeared opaque, *i.e.*, less translucent, in areas, and had a slightly crusted surface. The skin maintained with cortisone incorporated in the medium appeared slightly shrunken but still quite normal. At this time, most of the treated grafts were transplantable, but less than one half of the control grafts could be autografted successfully.

After 3 weeks in culture, marked changes were observed in the untreated skin. Most of the control cultures appeared to have degenerated severely and none survived as autografts (fig. 6). In contrast, although the cortisone-treated skin was shrunken and altered in appearance, it seemed viable (fig. 7). Of the 12 treated cultures that were subsequently grafted, 10 survived.

After 4 weeks *in vitro*, both the treated and untreated skin had degenerated. The skin of the controls appeared to be little more than dry, eschar-like material and the treated skin was extremely friable. However, 2 of the 12 treated cultures were successfully grafted.

### Microscopic Observations

Skin that is obtained from the auricular area of a strain A mouse has notably thin, even epithelium with no definite rete pegs. There are many sebaceous glands in the dermis and a characteristically uniform fatty layer (fig. 9). Examination of serial sections revealed that frequently the fatty layer was not obtained continuously in the grafts because of the mechanical difficulty in the removal of such thin skin evenly and accurately.

Histologic examination of the control cultures fixed after 1 week *in vitro* revealed that some changes had occurred (fig. 10). The connective-tissue matrix appeared to be appreciably thinner in all the specimens examined. The sebaceous and sweat glands and hair follicles were beginning to degenerate. The epidermis and dermis were usually adherent, but some loss of cellularity in the dermis was observed. Sections of skin maintained with cortisone acetate for 1 week appeared essentially normal (fig. 11). Generally the epidermis and dermis were adherent and in good condition. The adnexa appeared normal and no changes in the connective-tissue matrix were observed.

After 2 weeks *in vitro*, the untreated skin was markedly altered, particularly in the dermis (fig. 12). The number of cells and the amount of matrix in the connective tissue had appreciably diminished. In many areas the epidermis and dermis were no longer adherent and only vestiges of the glands and hair follicles remained. There was general loss of the normal cell patterns as both necrotic cells and primitive cell types became increasingly prominent. However, some areas of epithelium and all the fatty layers seemed normal. In contrast, the cortisone-treated skin still appeared quite normal. Sections taken at 2 weeks generally showed some thinning of the dermal matrix, but the cells appeared normal in configuration and number (fig. 13). The epidermis and dermis were intact and adherent in most areas. The glands and hair follicles had begun to show signs of degeneration.

An interesting ancillary effect was observed in some control cultures after 2 weeks and in many, after 3 weeks *in vitro*. Small spheres of embryo-like tissue were often seen attached to the lower, dermal surface of the skin. When these spheres were sectioned, they were composed of viable primitive, predominantly fibroblastic, cell types. The centers of the spheres were sinusoid and might be composed of atypical adipose tissue (fig. 8).

After 3 weeks in organ culture the skin of the control was an escharic shell (fig. 14). The dermis was sparsely populated with cells and the fatty layers had deteriorated. Obviously, the control cultures would not function as intact grafts. After the same period in culture, the skin treated with cortisone still retained many normal-looking areas (fig. 15). In many sections, however, the epidermis and dermis were no longer adherent. In many specimens the matrix of the dermis was quite thinned and, in general, was sparsely populated with cells. Remnants of the adnexal organs remained. Based on our experience, the skin from which the section shown in figure 15 was taken could have been successfully autografted.

Sections of skin from the control, examined after 4 weeks in culture, showed that only an eschar-like residue and occasional spheres of embryonic cells remained (fig. 16). A luxuriant growth of primitive cells was regularly seen on the floors of the flasks at this time. None of the control cultures could be successfully grafted at this stage. After 4 weeks in organ culture, the skin treated with cortisone still retained some degree of cellularity in the dermis and viable areas of epithelium were seen (fig. 17). However, all vestiges of adnexal organs had disappeared and the skin was generally in poor condition. Some degree of organization was still evident after 5 weeks *in vitro*.

Sections stained by the Masson technique confirmed that there was a generalized progressive loss of the connective-tissue matrix and cells in both the control and treated skin cultures. This process was decelerated appreciably by the addition of cortisone acetate.

## DISCUSSION

From the results, it is apparent that cortisone acetate is effective in slowing the processes which are responsible for the degenerative modifications of organized skin *in vitro*. It appears that the over-all changes which occur are twofold in character. At first, many cells in the tissue become modulated into more primitive nonfunctional forms that migrate readily from the graft. Then, gradually, the connective-tissue matrix becomes thin. The loss of matrix could be due to decreased production from the cells, to an accelerated lytic process, or to a decrease in the number of cells in the dermis because of cell migration. Probably the accelerated loss of the adnexal organs in the skin cultures of the control is explained by the more rapid loss of the connective-tissue elements

necessary for nourishment and support of the adnexa. Two phenomena which may signal the degree of cell modulation are 1) the luxuriant halo of modulated cells on the floors of the flasks of control organ cultures, and 2) the spheres of embryonic tissue attached to the dermis of the control cultures.

Cortisone retarded the disorganizing phases. The treated skin retained its cellularity and connective-tissue matrix for longer periods in culture than did the skin of the control. Halos of cells were not found in the treated cultures and spheres were not seen until the skin had been in culture for 4 weeks. These observations help to explain the fact that skin treated with cortisone survives for longer periods *in vitro* and can be more frequently autografted with success than the untreated skin. Since the regressive processes are only slowed by the addition of cortisone acetate, it will be interesting to see if other steroids may be more effective in extending the time that skin can be maintained in organ culture. Further studies on steroid compounds are in progress.

Another aspect of this research worth exploring is the use of this organ-culture system for testing the effects of other chemical agents *in vitro*. It is possible to observe the action of physiologic agents, either topically applied or incorporated into the medium, on the intact skin without the influences of the humoral mechanisms operating *in vivo*. Observations made in culture may be subsequently tested *in vivo* with a simple autografting procedure.

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# PLATES

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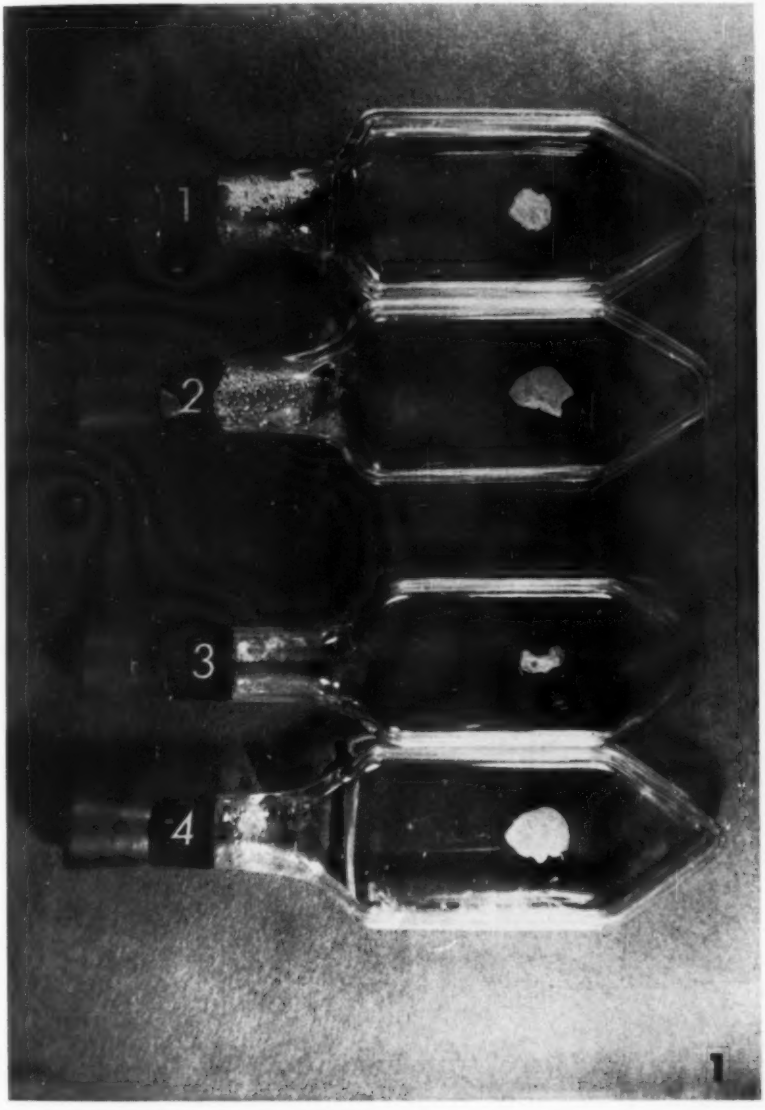
PLATE 176

FIGURE 1.—Two pairs of organ cultures of auricular skin of mice after maintenance for *3 weeks in vitro*. Flasks 1 and 3 contain untreated controls. Flasks 2 and 4 contain skin from opposite ears of the same two animals, treated *in vitro* with cortisone acetate. × 1



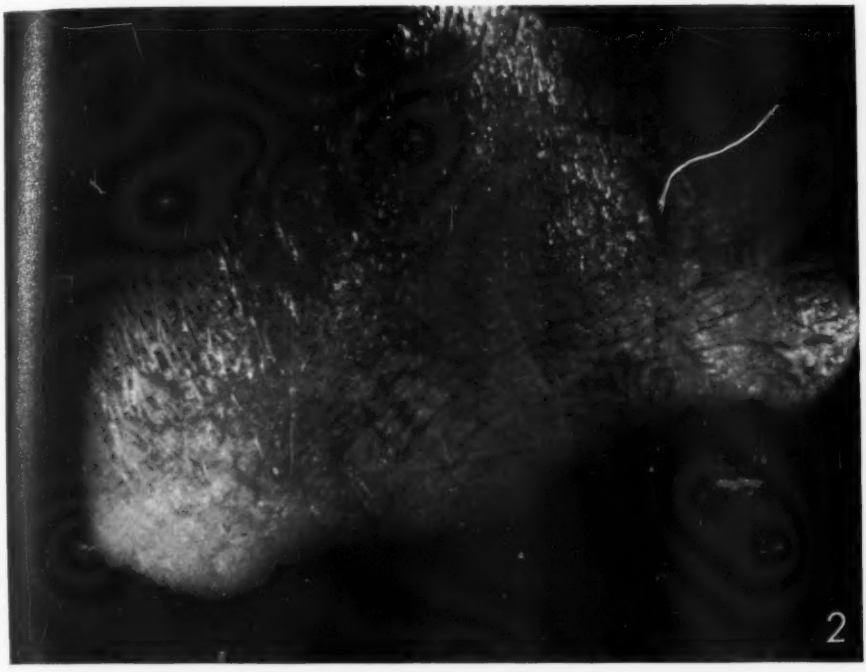



607399—61——8

PLATE 177

FIGURE 2.—Full-thickness auricular skin from a strain A mouse, floating on fluid medium, immediately after placement in culture. Photographs in this series (figs. 2 through 7) were taken through the glass culture flasks. × 8

FIGURE 3.—Full-thickness skin from opposite ear of same mouse immediately after placement in culture. Cortisone acetate was added to flask. × 8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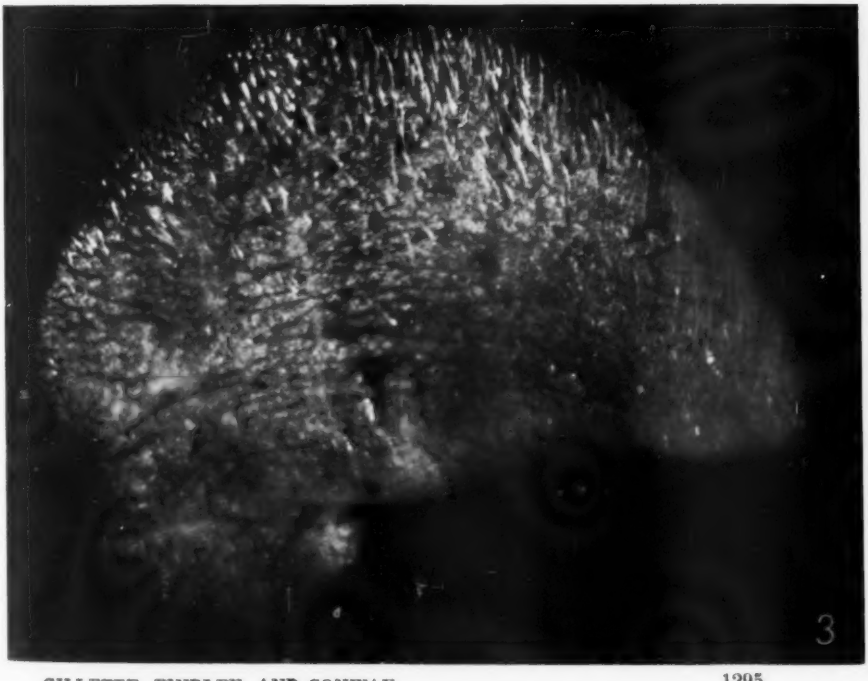
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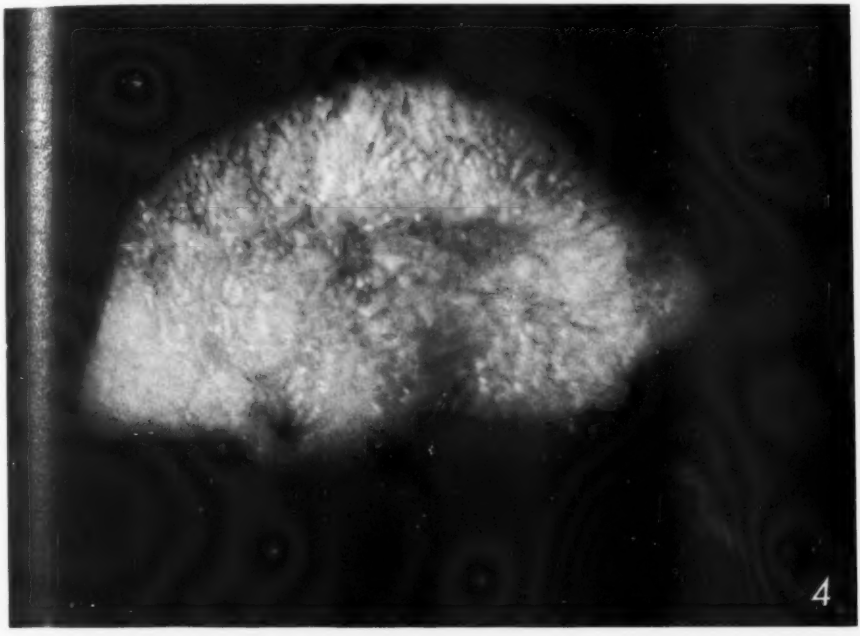
PLATE 178

FIGURE 4.—Control organ culture shown in figure 2 after *1 week in vitro*. *Note* marked shrinkage and some texture changes. × 8

FIGURE 5.—The cortisone-treated organ culture shown in figure 3 after *1 week in vitro*. The architecture of the skin has not changed observably. × 8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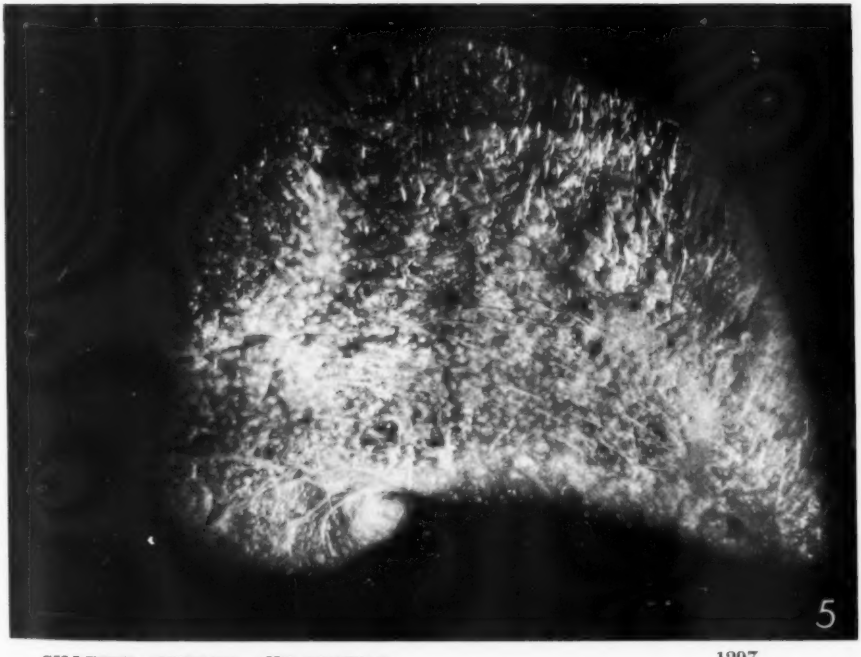
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PLATE 179

FIGURE 6.—Control organ culture after *3 weeks in vitro*. Marked shrinkage is evident and hair pattern is patchy. Skin is opaque, scaly, fragile, and could not be grafted successfully. × 8

FIGURE 7.—Cortisone-treated culture after *3 weeks in vitro*. Some shrinkage and increased opacity are observable. Degenerative changes are apparent but hair pattern appears normal. This skin would probably function as an intact graft. × 8

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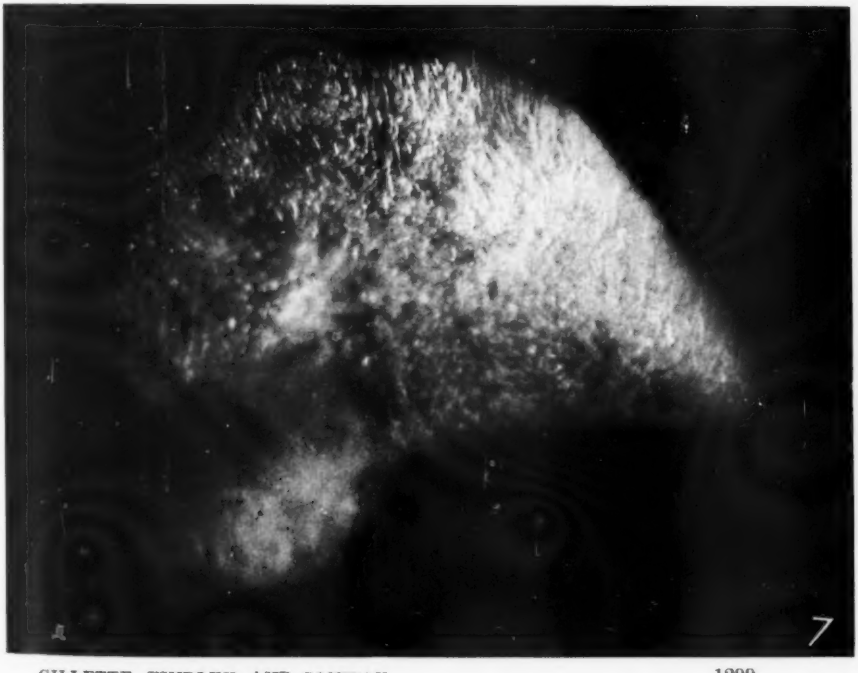
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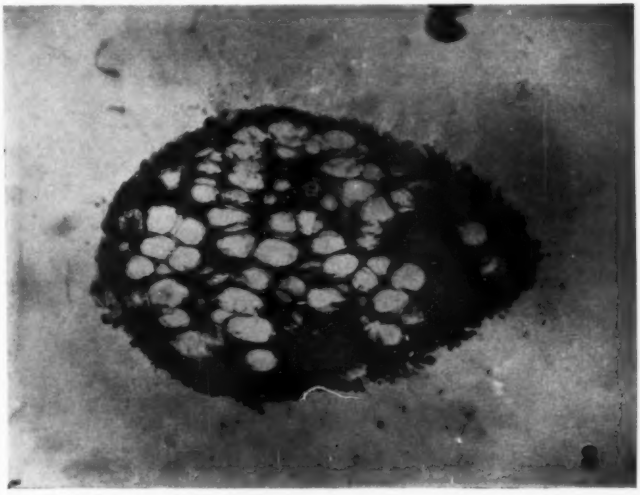
PLATE 180

FIGURE 8.—Typical sphere of modulated cells that appears frequently in untreated cultures after *2 or 3 weeks in vitro*. Hematoxylin and eosin. × 100

FIGURE 9.—Biopsy of normal auricular skin from strain A mouse. Hematoxylin and eosin. × 160



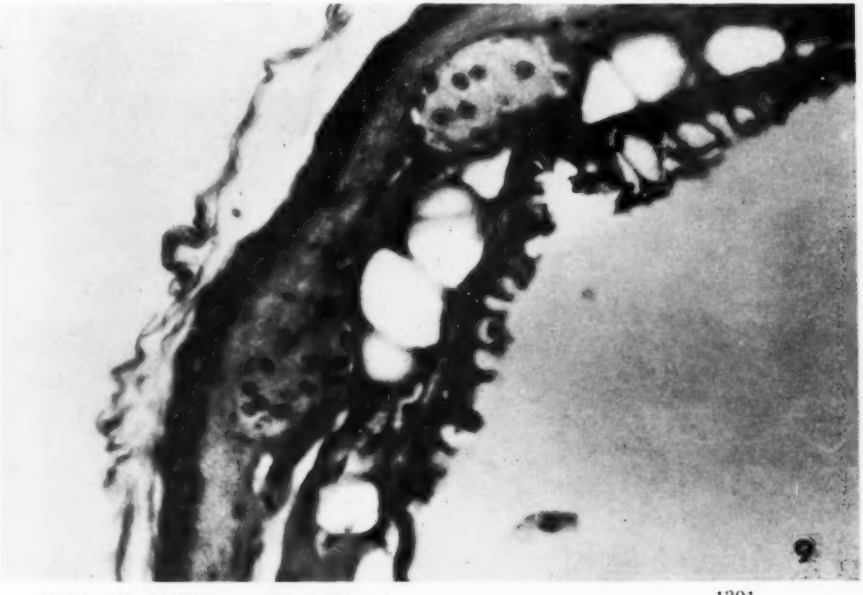


PLATE 181

FIGURE 10.—Untreated skin from strain A mouse after *1 week* in organ culture. *Note* thinning matrix and decreased cellularity of connective tissue, some epithelial slough, and cell migration. Hematoxylin and eosin. × 160

FIGURE 11.—Skin from opposite ear of same animal after *1 week* in organ culture with cortisone. The connective tissue appears normal. There is some epithelial slough, but the rending of epithelium from connective tissue seen in some sections is largely an artifact produced by the effect of routine histologic procedures on the delicate specimens. Hematoxylin and eosin. × 160

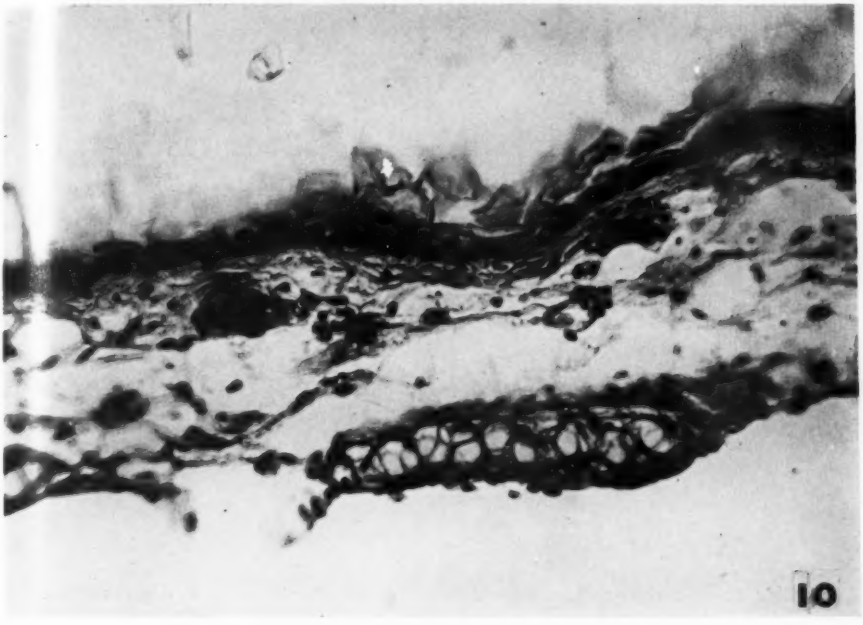

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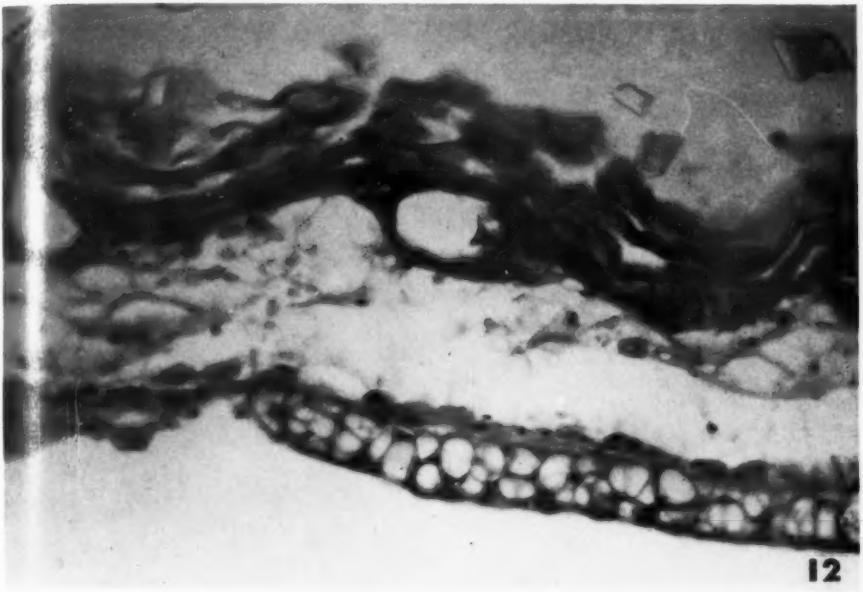
PLATE 182

FIGURE 12.—Untreated skin from strain A mouse after *2 weeks* in organ culture. *Note* deterioration of connective tissue and hair follicles. Hematoxylin and eosin. × 160

FIGURE 13.—Skin from opposite ear of same animal after *2 weeks* in organ culture with cortisone. *Note* good condition of connective tissue and adnexa. Beginning disorganization and cell migration in epithelium can be seen. Hematoxylin and eosin. × 160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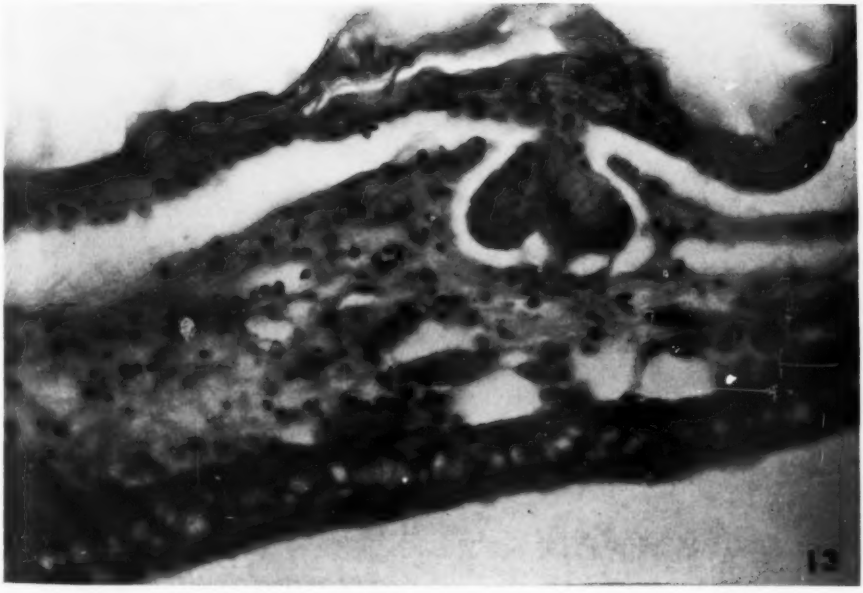
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PLATE 183

FIGURE 14.—Untreated skin from strain A mouse after *3 weeks* in organ culture. Only a small network of connective-tissue cells remains and there appears to be no viable epithelium. Hematoxylin and eosin. × 160

FIGURE 15.—Skin from opposite ear of same animal after *3 weeks* in organ culture with cortisone. Probably this skin would have been a successful autograft. Hematoxylin and eosin. × 160

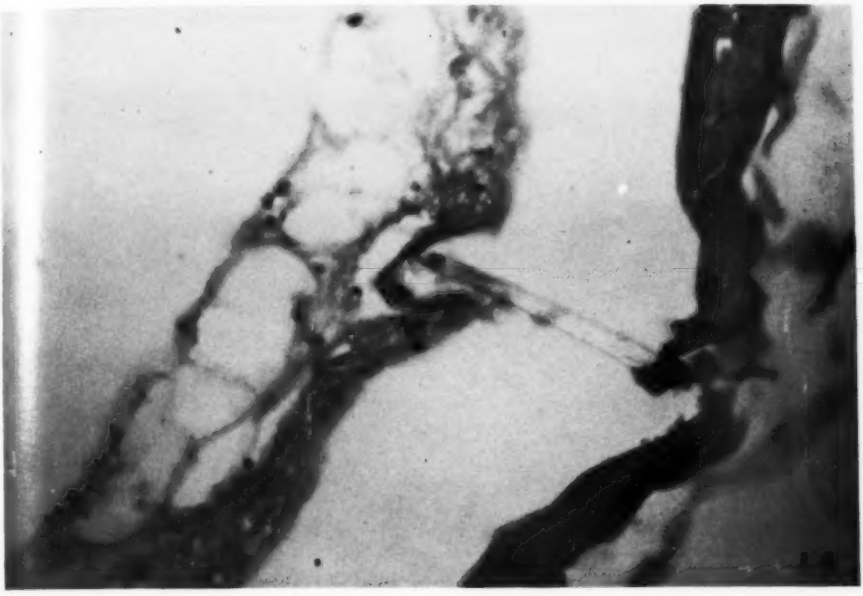

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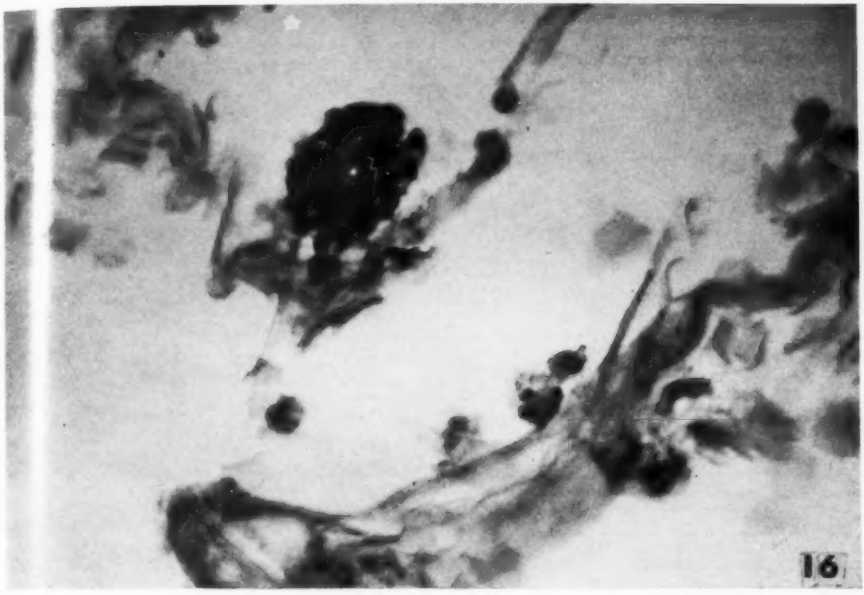
PLATE 184

FIGURE 16.—Untreated skin from strain A mouse after *4 weeks* in organ culture. Only scattered viable cells remain. Hematoxylin and eosin. × 160

FIGURE 17.—Skin from opposite ear of same animal after *4 weeks* in organ culture containing cortisone. *Note* thin layer of viable epithelium, loss of cellularity and matrix in connective tissue, and deterioration of sebaceous gland. Hematoxylin and eosin. × 160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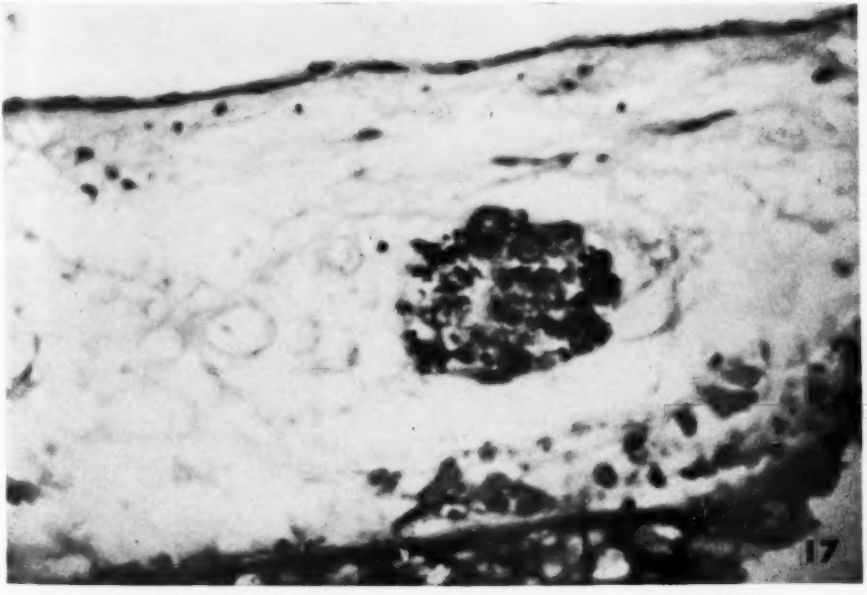
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# Studies on the Effects of Surgery on Transplanted Animal Tumors and Their Metastases [1]

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SUMMARY

**The effects of amputation of the primary tumor on the development of metastatic lung tumors were studied in 3 transplanted mouse tumor systems. Complete amputation of the primary tumor sharply reduced the number of metastatic foci. Controls had numerous small foci, but amputees had fewer but larger foci. Since amputees live longer, their lung tumors eventually became extremely large and sometimes completely covered the surface of the lungs. Incomplete removal of the primary tumor did not significantly alter the number of metastatic foci. Instead, it yielded results similar to those for unamputated controls, which suggested that surgical trauma *per se* does not result in enhanced formation of metastases. Amputation of the primary tumor also reduced the number of lung metastases in both aged mice (1 year) and relatively young mice (6 and 12 weeks). A lesser rate of primary tumor growth was noted for the aged host.—J. Nat. Cancer Inst. 27: 1311–1321, 1961.**

MODERN CANCER therapy, while not always affording a cure, has a palliative effect and often allows cancer patients to live longer. This should aid cancer therapists in further understanding the bizarre metastatic behavior sometimes exhibited by malignancies. However, basic conclusions are often invalid because the types of human tumors are so varied and there are not enough untreated controls.

Using previously standardized tumor-host systems in animals, one can study, under rather unique circumstances, alterations in behavior of the tumor. This experimental approach was well recognized, studied, and reported by investigators, such as Clunet (*1*), Ehrlich (*2*), Tyzzer (*3*), Wood (*4*), in the early 1900's. Recent literature abounds with reports concerning a variety of procedures applied to the host or its primary tumor (*5-9*).

This study attempts to explain more fully the effects of removal of a primary tumor on the size and number of metastases that subsequently develop. It describes the alterations in metastases as related to: (*a*) life-

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span of an animal after complete removal of the primary tumor, (*b*) partial removal of the tumor, and (*c*) the age of the animal.

## MATERIALS AND METHODS

Three tumor-host systems were used: Cloudman S91 melanoma grown in (C × DBA/2)$F_1$ hybrids; Sarcoma DBA49 in strain DBA/P mice, and Lewis sarcoma T241 in strain C57BL/6JN mice. The techniques have been previously described (*8*, *9*).

The tumorous material was injected into the right hind-leg muscle of 6-week-old mice and serially transferred by the cytosieve technique. One g of viable tumor was removed aseptically from the donor mouse, mixed with 10 ml of normal saline, passed through the Snell cytosieve (*10*), and then 0.05 ml of the mixture was injected into the muscular portion of the hind leg of the recipient mouse. Except for that part of the study related to mouse age, recipients were 6-week-old female mice. Uniformly, these procedures resulted in the successful appearance of "primary" tumors, of which all grew progressively and none spontaneously regressed.

In studies in which the leg with the primary tumor was amputated, the cautery-clamp technique was used. The local recurrence rate of the tumor was less than 2 percent. When the leg bearing the tumor was partially amputated, an incision was made and the bulk of the gross tumor was curetted. The edges of the wound were then closed with clips.

The day after amputation, the mice were caged individually and provided with food and water *ad libitum*. Such individual housing improves the longevity of mice. At specified times after the amputation, groups of mice were killed and examined. Since the unamputated controls had the shortest survival period, the dates of examination were determined by this factor, which permitted a valid comparison of results for amputated and control groups. Examination revealed metastases only in the lungs. These foci were counted and "sized" as previously described. The surface tumors were counted and "sized" with the use of a 3-D lens magnifier by comparison with sizes of standard circles drawn to a scale constructed so that the diameter of the smallest circle was 50 $\mu$, and the increment between each successive size was 50 percent (*9*).

Three groups of experiments were performed:

1) Studies of the progressive development of pulmonary metastases after removal of the primary tumor. (The increased longevity of mice, when their primary tumors were amputated, made it possible to observe them for longer periods than in earlier work in which mice were observed for the shorter period in which controls survived.)

2) Studies of the effect of partial amputation on occurrence of pulmonary tumors in contrast to complete removal of the primary tumor.

3) Studies demonstrating the effects of amputation of a primary tumor in mice of different ages. In these experiments results for 6-week-old and 3-month-old mice are compared. Data are also obtained for 1-year-

old mice, but later amputation and dates of killing were used. The better longevity for 1-year-old controls made the delay of killing feasible. Both old and young recipients were inoculated with the same tumor material on the same day.

In all studies, mice were randomly assigned to the various treatment groups. Animals were received in the laboratory as weanlings and were kept in colonies until assigned to an experiment.

## RESULTS

Tables 1 to 3 show summary data for the various experiments. These data include for each experimental group: median number of lung tumors per mouse; median number of lung tumors per tumor-bearing mouse; percentage of mice with any lung metastasis; average size of lung metastases; and the number of mice in the experiment.

### Progressive Development of Pulmonary Metastases After Complete Primary-Tumor Removal

The data for table 1 (2 experiments combined for each system) extend for 3 weeks post amputation for the T241 sarcoma, 4 weeks for the DBA49 sarcoma, and 9 weeks for the S91 melanoma. In all instances this is beyond the time that unamputated controls would have survived. For

TABLE 1.—Progressive development of pulmonary metastases after amputation of primary tumor

| T241 sarcoma in the C57BL/6JN mouse (amputation 10 days post tumor inoculation) | | | |
|---|---|---|---|
| Weeks post amputation: | 1 | 2 | 3 |
| Median number lung tumors per mouse | 2 | 8 | 8* |
| Median number lung tumors per tumor mouse | 3 | 8 | 8* |
| Percent of animals with metastases | 75 | 100 | 100 |
| Average size of metastatic foci | 7.0 | 9.2 | 12.2* |
| Number of mice | 47 | 47 | 44 |

| DBA49 sarcoma in the DBA/P mouse (amputation 14 days post tumor inoculation) | | | |
|---|---|---|---|
| Weeks post amputation: | 1 | 3 | 4 |
| Median number lung tumors per mouse | 9 | 17 | 12 |
| Median number lung tumors per tumor mouse | 10 | 17 | 12 |
| Percent of animals with metastases | 93 | 100 | 100 |
| Average size of metastatic foci | 6.4 | 6.9 | 11.0 |
| Number of mice | 46 | 49 | 48 |

| S91 melanoma in the (C × DBA/2)$F_1$ hybrid (amputation 21 days post tumor inoculation) | | | | | |
|---|---|---|---|---|---|
| Weeks post amputation: | 1 | 3 | 5 | 7 | 9 |
| Median number lung tumors per mouse | 0 | 3 | 6 | 4 | 4* |
| Median number lung tumors per tumor mouse | 4 | 7 | 9 | 7 | 5* |
| Percent of animals with metastases | 34 | 72 | 88 | 87 | 88 |
| Average size of metastatic foci | 2.3 | 4.9 | 7.8 | 9.8 | 9.5* |
| Number of mice | 49 | 49 | 49 | 47 | 49 |

*Does not include mice in which the lungs were overgrown with extremely large tumors. There were 10 such mice in the T241 group; 11 in the S91 group.



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all 3 tumors there is a pattern of moderate increase in the number of lung metastases per mouse, some increase in the percentage of mice with such metastases (most notably for the S91 melanoma), and pronounced increase in the average size of these metastases. In the final groups that were killed for the T241 sarcoma and the S91 melanoma, pulmonary tumors in many mice were large and confluent, the lung surface often completely covered by tumor.

The increased number of tumors in the later weeks, however, remained moderate in magnitude and less than those ordinarily found in unamputated controls at an earlier time. The median number of 8 such tumors for the T241 sarcoma, 3 weeks after amputation and 31 days after inoculation, contrasts with 60 tumors in control mice killed a week earlier (table 2) and 46 for the 6-week-old controls (table 3). Similarly, for the DBA49 sarcoma, the median number of 12 tumors 4 weeks after amputation contrasts with 30 tumors in earlier controls (table 2) and 13 in 6-week-old mice (table 3). Finally, for the S91 melanoma, the median number of tumors found 4 to 9 weeks after amputation compares with 6-week earlier control counts of 9 tumors (table 2) and 13 tumors (table 3).

## Effects of Partial Amputation

Except for inclusion of the partial-amputation groups, these experiments were conducted in the manner previously reported. Insofar as the effects of amputation are concerned, the results of table 2 (2 experiments combined for each system) confirm previous reports (*8*, *9*). Amputation

TABLE 2.—Effect of complete or partial amputation of a primary tumor on pulmonary metastases

| | Complete amputation | Partial amputation | Controls |
|---|---|---|---|
| **T241 sarcoma in the C57BL/6JN mouse** (amputation 10 days post tumor inoculation; killed 24 days post tumor inoculation) | | | |
| Median number lung tumors per mouse | 11 | 52 | 60 |
| Median number lung tumors per tumor mouse | 11 | 52 | 60 |
| Percent of animals with metastases | 100 | 100 | 100 |
| Average size of metastatic foci | 8.6 | 6.9 | 5.8 |
| Number of mice | 55 | 61 | 54 |
| **DBA49 sarcoma in the DBA/P mouse** (amputation 14 days post tumor inoculation; killed 28 days post tumor inoculation) | | | |
| Median number lung tumors per mouse | 9 | 29 | 30 |
| Median number lung tumors per tumor mouse | 10 | 29 | 30 |
| Percent of animals with metastases | 87 | 98 | 100 |
| Average size of metastatic foci | 7.3 | 7.1 | 6.0 |
| Number of mice | 62 | 61 | 59 |
| **S91 melanoma in the (C × DBA/2)$F_1$ hybrid** (amputation 21 days post tumor inoculation; killed 42 days post tumor inoculation) | | | |
| Median number lung tumors per mouse | 5 | 8 | 9 |
| Median number lung tumors per tumor mouse | 7 | 10 | 11 |
| Percent of animals with metastases | 72 | 87 | 93 |
| Average size of metastatic foci | 3.5 | 2.9 | 2.7 |
| Number of mice | 52 | 52 | 51 |

reduced the number of metastatic lung tumors and increased their average size. There are also some indications of a reduction in the percentage of mice with such tumors.

This table shows that partial amputation of the primary tumor did not have a stimulating effect on metastasis, nor did it result in an inhibiting effect. Substantially, all results for the partial-amputation groups are similar to those for the unamputated controls, but differ from those for completely amputated groups.

### Effects of Mouse Age

Table 3 (combining 2 experiments for S91 sarcoma, single experiments for other systems) reveals few differences in the results for 6-week-old and 3-month-old mice. With DBA49 sarcoma, there is some indication that metastatic tumors are more numerous in older mice. This may only reflect the abnormally low incidence of such tumors in the 6-week-old mouse (*cf.* results for the corresponding groups of tables 1 and 2). For both the younger and older groups, the contrast in the results between the mice that were amputated and the controls is almost identical.

Results for mice of advanced age (1 year) are not directly comparable with those for the 2 younger age groups. The longer survival of the older mice, when their primary tumors were not removed, made it possible for us to delay killing mice with T241 and DBA sarcomas for an additional week and an additional 3 weeks for those with S91 melanoma. Amputation was also performed at a later time (4–7 days) in these older mice. At that time the primary tumors in the 1-year-old mice were comparable in size to those in the young mice at their earlier amputation time. The usual pattern of reduced metastatic tumor incidence and increased average tumor size after amputation is evident from the data for the 1-year-old mouse.

## DISCUSSION

Removal of the primary tumor leads to cessation of shedding of tumor cells and virtual termination of additional metastatic pulmonary-tumor formation. This is demonstrated by the great reduction in the number of pulmonary metastases and by the absence of small metastases after amputation. The current findings show that the number of metastases remains depressed for extended periods after amputation, while the average size of metastases continues to increase. This increase in size is caused partly by the disappearance of small metastases, as previously reported (*9*), and partly by an actual excess in the number of large metastases. That amputated and control mice show markedly different lung metastasis is vividly demonstrated in figures 1 and 2. The lung from a primary-tumor-bearing control (fig. 1) shows pulmonary-tumor foci of various sizes—so numerous they could not be counted. Figure 2, in sharp contrast to figure

TABLE 3.—Studies with young and old mice on the effect on pulmonary metastases of amputation of a primary tumor

| | 6-Week-old mice | | 3-Month-old mice | | 1-Year-old mice | |
|---|---|---|---|---|---|---|
| | Amputated | Control | Amputated | Control | Amputated | Control |
| **T241 sarcoma in C57BL/6JN mouse** | | | | | | |
| Amputation day post tumor inoculation | 10 | | 10 | | 14 | |
| Day killed post tumor inoculation | 24 | | 24 | | 31 | |
| Median number lung tumors per mouse | 9 | 46 | 11.5 | 52 | 19 | * |
| Median number lung tumors per tumor mouse | 10 | 48 | 14 | 53 | 20 | * |
| Percent of animals with metastases | 96 | 96 | 96 | 100 | 100 | 100 |
| Average size of metastatic foci | 7.1 | 3.8 | 7.0 | 4.8 | 8.6 | 6.2 |
| Number of mice | 27 | 25 | 26 | 26 | 24 | 25 |
| **DBA49 sarcoma in DBA/P mouse** | | | | | | |
| Amputation day post tumor inoculation | 14 | | 14 | | 21 | |
| Day killed post tumor inoculation | 28 | | 28 | | 35 | |
| Median number lung tumors per mouse | 3 | 13 | 11 | 25 | 19 | 41 |
| Median number lung tumors per tumor mouse | 3 | 14 | 13 | 26 | 20 | 41 |
| Percent of animals with metastases | 90 | 90 | 90 | 100 | 95 | 100 |
| Average size of metastatic foci | 6.5 | 6.5 | 6.5 | 6.2 | 8.9 | 7.1 |
| Number of mice | 24 | 26 | 27 | 25 | 29 | 27 |
| **S91 melanoma in (C × DBA/2)$F_1$ hybrid** | | | | | | |
| Amputation day post tumor inoculation | 21 | | 21 | | 28 | |
| Day killed post tumor inoculation | 42 | | 42 | | 63 | |
| Median number lung tumors per mouse | 1 | 4 | 2 | 6 | 4 | 13 |
| Median number lung tumors per tumor mouse | 5 | 7 | 4 | 8 | 7 | 14 |
| Percent of animals with metastases | 53 | 76 | 67 | 86 | 77 | 92 |
| Average size of metastatic foci | 4.1 | 2.5 | 4.4 | 2.3 | 7.7 | 3.3 |
| Number of mice | 46 | 45 | 44 | 44 | 48 | 46 |

*Foci were too numerous to count accurately; average tumor size is based on a representative portion of the foci observed.

1, shows the lung from an animal with the primary tumor removed in which there are only large tumor deposits and no small metastases.

In no instance, with each of these 3 tumor systems, has metastasis to any organ or region other than the lungs been observed. Death is apparently due, in the control animal, to the combined effects of the large primary tumor and diffuse lung metastases. In the amputated animals, death has been associated solely with complete lung parenchymal replacement by tumor.

The relationship of the primary tumor to the increased growth of metastases already implanted is still unclear. Possibly the primary tumor in some way exerted an inhibiting influence on the progressive growth of the metastatic tumors. Alternatively, this growth reflected only the better nutritional status of mice after the primary tumors were removed. The relationship of tumor growth to nutritional intake has been reported by McEuen *et al.* (*11*) and Bischoff *et al.* (*12*).

The great number of metastatic foci in the control animals further suggests that reduced competition between foci may be a factor responsible for the massive growth of the large foci in the amputated host. The many individual pulmonary deposits found in the unamputated controls may compete with one another for nutrient material and space in which to grow, and consequently cannot become significantly large before the animal dies with a bulky primary tumor.

In contrast to the present findings, other investigators (*5–7*) have reported an increase in lung metastases associated with amputation of primary tumor. This could be due to one or all of the following causes: 1) failure to use techniques capable of detecting the many tiny foci, such as those found only by dye insufflation of the lung, with magnification being used during the counting process; 2) failure to make counts for amputated and control animals on comparable days, *i.e.*, the greater longevity for amputated mice caused counts to be made at a later time; 3) variance between tumor-host systems. With our experimental procedures, metastases have consistently diminished in amputees.

The effects noted for partial amputation are of particular interest. Typical behavior of the primary tumor after partial amputation was as follows: After the bulk of the gross tumor was actually removed, there was a period of quiescence, which lasted 3 to 6 days. During this period no growth of tumor was noted in the partially amputated leg. A period of rapid recurrent growth followed, and the recurrent primary tumors attained sizes comparable to those in the controls that had not had amputation.

It seems logical that a tumor can grow only as rapidly as its blood supply will allow. The vascular supply was temporarily interfered with by curettement, thus delaying the regrowth of tumor, but after regeneration the regrowth was rapid until its growth became similar to that of the control series. If there was an immunological response of the host to the sudden showering of tumor emboli at curettement, it was quickly lost.

With metastatic foci numbers, partial amputees were substantially identical to the controls with no amputation. There was no evidence that partial amputation resulted in a reduction. Neither was there evidence that the trauma of surgery led to an increase in foci numbers, as suggested by others (*3*). Instead, the data suggest that the continued existence of a primary tumor is a dominant factor in determining the extent to which metastatic foci occur. There is some evidence from the data that the average size of metastatic foci is larger after partial amputation than after removal of the primary tumor, but not as large as after complete amputation. The technique of curettement employed may be responsible. If the massage accompanying curettement caused shedding of metastatic cells and subsequent metastatic tumor formation, the resulting tumors would have been, on the average, older, and therefore, larger than those in the controls.

In this connection, Zeidman (*13*) suggested that the longer a primary tumor exists in the host, the greater the number of tumor emboli released and the greater the number of metastases. He did not report any significant correlation between the number of pulmonary metastases and the final size of the primary tumor.

The data concerning 1-year-old mice in table 3 cannot be directly compared with the 6-week-old or 3-month-old mice or with Zeidman's work, because, even though the primary tumors were left in the host 4 to 7 days longer and reached the usual 1.5 to 2 cm for amputation, these animals were killed 7 to 21 days later. Thus it cannot be stated whether the increased size and growth of metastases was due to the time the primary tumor remained in the host or the time the host was allowed to live before it was killed.

The effects of amputation in the older mouse present two interesting points. In aged mice (1-year-old) amputation produced the same phenomenon of fewer but larger metastatic foci that had been observed in younger animals. This phenomenon apparently is not peculiar to the young host. Also, the primary tumor grew more slowly in the aged host than in the young host, and it was for this reason that the older animal was amputated at a later time. Since older animals have a slower rate of metabolic activity, it is likely that this is a factor in the slower growth of the primary tumor.

Age of the host system is thus an added variable that must be considered in studies of tumor-host systems. The response of the host to a transplanted tumor is dependent on numerous factors: manner of preparation of the inoculum (cytosieve or trocar, site and manner of inoculation, intravenous, arterial, or intramuscular), amount of inoculum, viability of the tumor cells inoculated, the housing of the animals (colony or individual), and the age of the host system.

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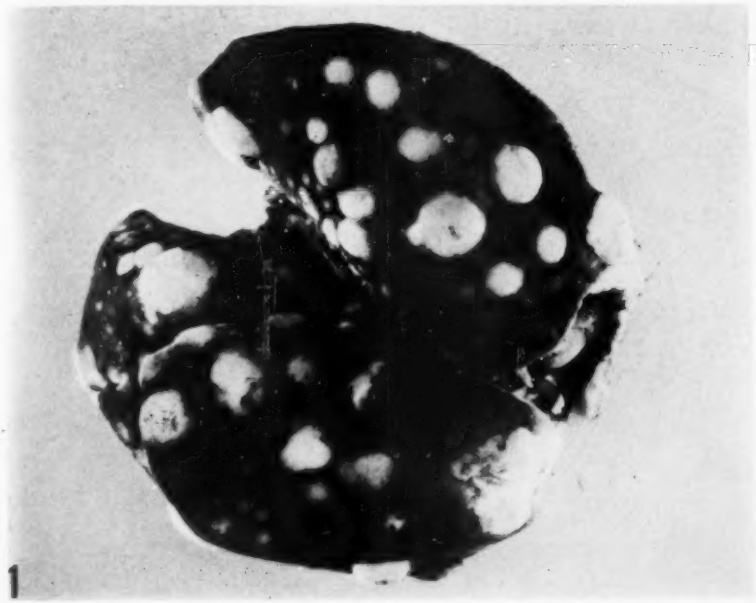
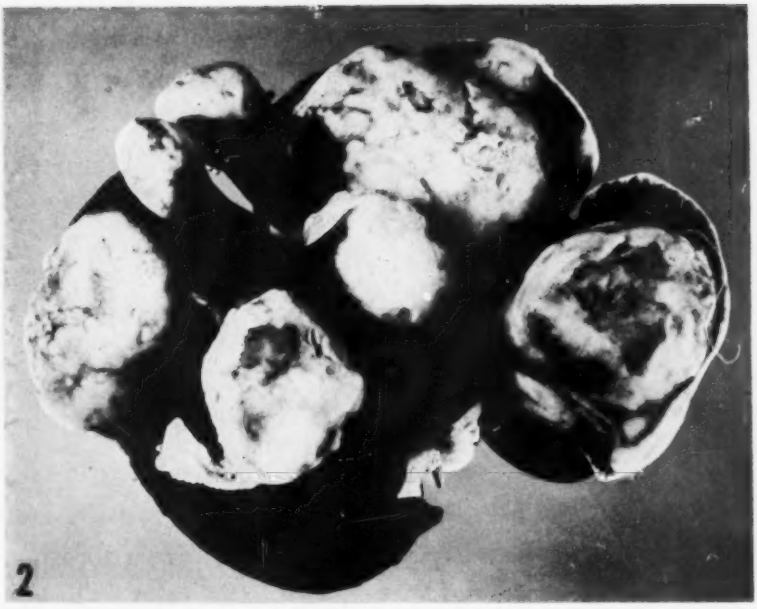
PLATE 185

FIGURE 1.—Small metastatic lung tumors in dye-insufflated lung taken from a 1-year-old control mouse with primary T241 sarcoma left intact. Killed on postinoculation day 31.

FIGURE 2.—Large metastatic lung tumors in dye-insufflated lung taken from a 1-year-old mouse with primary tumor completely removed 14 days after inoculation. Killed on postinoculation day 31. *Note* absence of small metastatic foci.






607399—61——10

# Transplantable Tumors of the Syrian Golden Hamster (*Mesocricetus auratus*) [1,2,3,4]

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SUMMARY

**The biological behavior and morphological features of 8 transplantable tumors of the Syrian golden hamster are described. Included are a 20-carcinoma of the breast, an *N*-2-methylcholanthrene-induced adenofluorenylacetamide induced bile-duct carcinoma, and a cellular blue nevus-like melanotic tumor induced by the cutaneous application of 7,12-dimethylbenz[a]anthracene (DMBA). Five additional neoplasms, a plasmacytoma, a fibromyxosarcoma, a malignant neurilemmoma, a cutaneous adnexal cystadenocarcinoma, and a malignant hemangiopericytoma, occurred either in untreated controls or as single incidents in carcinogenic studies where no causal relationship could be established. Instances of transplantable hemangiopericytoma, adenocarcinoma of the breast, and cutaneous adnexal cystadenocarcinoma have not been reported previously in hamsters.—J. Nat. Cancer Inst. 27: 1323–1339, 1961.**

THE SYRIAN golden hamster (*Mesocricetus auratus*) (*1*) was introduced into the field of experimental biology in 1930 (*2*) and has been used since as an experimental animal for a variety of purposes. In the field of cancer research, it has been used for the testing of chemotherapeutic agents (*3*), in chemical carcinogenesis (*4, 5*), and in transplantation experiments, with particular reference to heterotransplantation (*6*).

Tumors of hamsters have been reported in the literature since 1939 when Gye and Foulds (*7*) described an induced spindle-cell sarcoma. The first spontaneous transplantable tumors, designated as "cortical hypernephromas of the adrenal" were reported by Ashbel in 1945 (*8*). Numerous other transplantable tumors have been recorded in the literature (*9–32*).

This report includes transplantable tumors originating from apparently spontaneous neoplasms, as well as from carcinogen-induced ones. Their biological behavior and morphological characteristics are described. All

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[2] This study was supported by grants CRT-5070 and CS-9212 from the National Cancer Institute, National Institutes of Health, Public Health Service.

[3] The tumors described in this paper are available to interested investigators.

[4] After this paper was submitted for publication, a report on a series of transplantable tumors of the Syrian hamster was published by Fortner, G. J., Mahy, A. G., and Schrodt, G. R., in *Cancer Research*, vol. 21, Supplement on Cancer Chemotherapy Screening Data X, 1961, pp. 161–198.



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represent well-established lines carried in not less than 6 generations. Three, an adenocarcinoma of the breast, a hemangiopericytoma, and a cutaneous adnexal cystadenocarcinoma, have not been reported previously.

## MATERIALS AND METHODS

The tumors appeared and were transplanted into hamsters originally obtained from Abrams Small Stock Breeders, Chicago, Illinois, and bred at random in our laboratory since 1958. The hamsters were housed in plastic cages, fed Rockland mouse diet, and given water *ad libitum*. Groups of males or females were used in successive transplantations but not necessarily in alternating sequence. The age of the recipients varied between 4 and 52 weeks. Two to 4 animals were used for each transplant.

Tumor tissue was obtained by biopsy or at postmortem examination and transplanted subpannicularly either by implantation of a small fragment or by inoculation of homogenized tissue, consisting of 1 part tumor and 2 parts saline, prepared in a glass tissue grinder. With both methods the number of takes, the latent period, and the growth pattern were similar. The lower back or the left flank of the animals was selected for the implantation. Every week the animals were weighed and the measurements of the tumors were recorded. Most animals were observed until death, or were killed when moribund. Some were killed earlier to obtain viable tissue for transplantation. All were autopsied. The tumor at the site of transplantation, the metastases, and any other gross lesions were studied histologically.

The induction of some tumors reported in this paper was achieved in experiments on chemical carcinogenesis in hamsters carried out by members of this laboratory (*33–35*).

## RESULTS

### Primary Adenocarcinoma of the Breast (LZ-6)

This neoplasm originated as a subcutaneous tumor (4 × 2 cm) in the right axillary region of a 42-week-old female that had received 3-methylcholanthrene by stomach tube (6 mg in 0.5 ml corn oil 3 times weekly, for 21 weeks) (*33*). It was first noticed in the 26th week of the experiment. The tumor was transplanted for 19 generations into 54 golden hamsters, 24 males and 30 females, and became palpable as a small nodule 2 to 4 weeks after transplantation into 50 animals (92.59%; average latent period: 2.3 weeks). The weight of the hosts bearing transplanted tumors increased on the average by 24.7 percent of the initial weight during the period of natural survival. The tumor-bearing animals died or were killed when moribund, between 2 and 10 weeks after appearance of the tumor (average: 4.5 weeks).

The implants grew evenly and constantly to a maximum size of 140

$\times$ 120 $\times$ 25 mm. They adhered to the skin and often ulcerated. Many areas of necrosis were scattered throughout the neoplastic tissue. The consistency was always soft. The sectioned surface varied from reddish to brown and showed many cystic cavities with hemorrhagic mucoid contents. The transplanted tumors did not appear to invade the surrounding tissues and metastases were not observed.

Microscopically the tumor was composed of numerous glandular formations showing marked variation in size and shape of the glandular acini. There were numerous intercommunicating cavities completely or partially subdivided by fibroepithelial papillary formations composed of a delicate vascular stroma and atypical neoplastic glands. In some areas the tumor had a more solid cellular appearance. The individual tumor cells showed pronounced variation in size and shape, and marked mitotic activity. The stroma was infiltrated with lymphocytes and plasma cells. The lumina of some of the acini were filled with a pink-staining precipitate (fig. 1). The microscopic appearance of the transplants was identical with that of the original tumor.

### Intrahepatic Bile-Duct Carcinoma (Cholangiocarcinoma) (LM-2)

This tumor appeared as a nodule 2 $\times$ 3.5 $\times$ 2 cm in the right lobe of the liver in a 47-week-old female after 31 weeks of treatment with *N*-2-fluorenylacetamide (0.4% solution in corn oil added to powdered diet) (*34*). It was transplanted for 10 generations into 33 golden hamsters, 15 males and 18 females. It took in 31 animals (96.96%), and became palpable as a small nodule 1 to 4 weeks after transplantation (average latent period: 2.7 weeks). The weight of the hosts decreased on the average by 49 percent of the initial weight from the time of transplantation until natural death. The animals died or were killed when moribund, between 4 and 20 weeks after appearance of the tumors (average: 11.3 weeks).

The implants grew evenly in all instances to a maximum size of 60 $\times$ 60 $\times$ 15 mm. Frequently ulcerated and with a thick capsule, they were well vascularized and showed a relatively small area of necrosis in the center of the neoplastic mass. The sectioned surface was partly solid and partly cystic; the color varied from dark red to reddish brown. The cysts were filled with hemorrhagic fluid. Grossly the tumors did not appear to invade the surrounding tissues. Metastases to the axillary and inguinal lymph nodes were observed in 8 animals (24.3%), and to the lungs in 2 animals (6.6%).

Microscopically the tumor was a well-differentiated adenocarcinoma of the bile-duct type (fig. 2). The lining cells of the ducts were columnar and their cytoplasm acidophilic. There was a tendency for the nuclei to be near the base. Secretory activity was manifested by the presence of "goblet cells" and mucus within the duct lumina. Evidence of invasiveness could be observed microscopically at the periphery of the tumor. The neoplastic ducts were separated by a moderately abundant fibrous stroma with areas of hyalinization.

### Cellular Blue Nevus-Like Melanotic Tumor (KW-2)

This neoplasm arose in the skin of the right flank of a hypophysectomized 65-week-old female golden hamster as a round, melanotic nodule 3 cm in diameter, 39 weeks after a single cutaneous application of 4 drops of a 1 percent solution of 7,12-dimethylbenz[*a*]anthracene (DMBA) in mineral oil. The tumor was transplanted for 6 generations into 24 golden hamsters, 10 males and 14 females; it took in 19 animals (79.2%) and became palpable as a small nodule 1 to 5 weeks after transplantation (average latent period: 2.6 weeks). The weight of the hosts bearing the transplants increased on the average by 24 percent of the initial weight during the period of natural survival. The tumor-bearing animals died, or were killed when moribund, between 10 and 30 weeks after the appearance of the tumor (average: 14 weeks).

The implants grew irregularly in most instances. Periods of growth alternated with periods of reduction in size. The maximum size recorded was 40 × 30 × 20 mm. Always well encapsulated, they were slightly vascularized and showed little necrosis. The consistency was always solid and the sectioned surface was black. The tumors did not appear to invade the surrounding tissues. Metastases were observed in the lungs in one instance.

Microscopically the original tumor was composed of a well-demarcated mass of heavily pigmented cells and had all the features previously described in carcinogen-induced melanotic lesions of the hamster (*36*). It occupied the deeper layer of the dermis and extended into the subcutaneous tissue. In one area it partly surrounded a distended hair follicle filled with desquamated keratin masses. Many tumor cells were so heavily pigmented that the cellular details were obscured. Extensive areas of necrosis were seen. The tumor was not like the malignant melanomas occurring spontaneously in this species (*23*), but closely resembled cellular blue nevi of man.

Sections from the first transplant gave a similar picture. The pigmentation was less pronounced than in the original tumor. There were moderate variations in size and shape of the tumor cells and many of them appeared to have delicate pigment-laden dendritic processes (fig. 3). Mitoses were present but not numerous. There was a scattering of heavily pigmented melanophages. In subsequent transplants cellular atypia and mitotic activity became increasingly more pronounced, while the estimated amount of pigmentation remained approximately the same. The microscopic features resembled those previously reported in a transplantable melanotic tumor originating in a nonhypophysectomized DMBA-treated hamster (*17*).

### Plasmacytoma (KG-13)

The tumor originated as a subcutaneous mass (1.2 × 2 cm) in the cervical region of an untreated 83-week-old male. It was transplanted for 9 generations into a total of 33 golden hamsters, 15 males and 18 females.

It took in 32 animals (96.96%) and became palpable as a small nodule 2 to 8 weeks after transplantation (average latent period: 4 weeks). The weight of the hosts bearing the transplants increased on the average by 5.4 percent of the initial weight during the period of natural survival. The tumor-bearing animals died or were killed when moribund, between 5 and 15 weeks after the appearance of the tumor (average: 6 weeks).

The implants grew regularly and constantly to a maximum size of 180 × 140 × 30 mm. They usually appeared encapsulated and slightly vascularized at the periphery. The inguinal, axillary, and cervical lymph nodes were involved in 6 animals (18.1% of the tumor-bearing animals), and the liver, spleen, and kidney in 1 animal each.

Microscopically the tumor was composed of relatively small cells with eccentrically located nuclei and relatively abundant cytoplasm. The nuclear chromatin was rather delicate, and single, small but distinct nucleoli were present in many nuclei. In some cells the nuclear membranes showed condensations of the nuclear chromatin on their inner surfaces, but only a few nuclei showed the spoke-wheel arrangement characteristic of mature plasma cells. A moderate number of binucleated forms were seen (fig. 4). There was pronounced mitotic activity. The periphery of the tumor was surrounded by a capsule of varying thickness consistent with the capsule of a lymph node, but no trace of pre-existing lymphatic tissue could be demonstrated in these sections. In one area the capsule was massively infiltrated by tumor cells. In the transplants the plasmacytic character of the cells was more striking than in the original tumor; the cytoplasm was more abundant and had a more distinctly basophilic staining quality. The eccentric location of the nucleus was also more apparent; however, the nuclei still lacked the characteristic spoke-wheel arrangement of mature plasma cells, which is entirely consistent with poorly differentiated plasmacytoma. Mitotic figures were numerous and atypical mitoses were observed. Necrosis, completely absent in the original tumor, was a conspicuous feature in the transplants.

### Malignant Neurilemmoma (LI-6)

This neoplasm originated as a subcutaneous nodule (2 × 2 cm) in the back of a 33-week-old female that had received 0.2 to 0.4 percent urethan in drinking water for 26 weeks (*35*). The tumor was transplanted for 9 generations into 28 golden hamsters, 14 males and 14 females. It took in 27 animals (96.42%). In the first generation the latent period varied from 3 to 10 weeks. In the following 6 generations the latent period shortened and the tumor became palpable as a small nodule 2 to 6 weeks after transplantation (average latent period: 3 weeks). The weight of the hosts bearing the implants increased by 20 percent of the initial weight during the period of natural survival. The tumor-bearing animals died or were killed when moribund, between 6 and 24 weeks after the appearance of the tumor (average: 11 weeks).

The implants grew to a maximum of 90 × 30 mm. They appeared

encapsulated and sometimes lobulated. They were slightly vascularized and showed a small amount of central necrosis. The sectioned surface was gray or grayish brown and predominantly solid, with a few cystic cavities which sometimes contained hemorrhagic fluid. Grossly the tumors did not appear to invade the surrounding tissues. Metastases to the lungs were observed in 5 animals (17.8% of the tumor-bearing animals).

Microscopically the tumor was composed of spindle-shaped cells with elongated and fusiform nuclei showing moderate variations in size and shape. The cytoplasmic borders were indistinct and the cytoplasm appeared to blend imperceptibly with a finely fibrillar matrix. Some areas were more cellular than others (fig. 5). In the less cellular areas the matrix had a bluish tint, suggestive of mucoid degeneration, in sections stained with hematoxylin and eosin. Organoid structures closely resembling Verocay bodies were a conspicuous feature (fig. 6). Viable tumor tissue was often limited to a zone in the immediate vicinity of blood vessels, while, more distant from them, areas of necrosis were observed. At the periphery a distinct capsule was present, but tumor cells appeared to have grown beyond the capsule and were seen in the pericapsular tissue. Relatively few mitoses were observed. While the tumor was relatively well differentiated and presented some of the characteristic features of an encapsulated neurilemmoma with well-developed Verocay bodies, the presence of large areas of necrosis and the pericapsular invasion, together with some cellular atypia, justifies the interpretation that it was a malignant variant of neurilemmoma. Reticulin stains showed an abundant, delicate fibrillar framework enveloping practically every cell of the tumor. The transplanted tumor differed from the original one by having more pronounced variation in size and shape of the nuclei, greater cellularity of the Verocay bodies, which were not as clear-cut as in the original tumor, and by more pronounced mitotic activity. The transplanted tumor showed an uninhibited invasion of host tissues with no evidence of encapsulation.

### Malignant Hemangiopericytoma (IZ-1)

This tumor was discovered as a 2 × 1 × 1 cm mass in the left flank of a 58-week-old female hamster. It invaded the diaphragm and the lower ribs, protruding into the peritoneal cavity. The animal belonged to a group that had received 0. 5 cm of a solution containing 10 percent carbon tetrachloride in corn oil by stomach tube once a week. The tumor appeared after 48 weeks of treatment. It was transplanted for 17 generations into 55 golden hamsters, 41 males and 14 females; the transplants grew in 53 animals (96.36% of the recipients).

In the first 5 generations, the latent period varied from 6 to 20 weeks (average: 9 weeks). In the following 9 generations the latent period was shorter, the tumors becoming palpable from 1 to 6 weeks after transplantation (average latent period: 3.5 weeks). The weight of the tumor-bearing hosts increased on the average by 32 percent of the initial weight during the period of natural survival. The tumor-bearing animals died

or were killed when moribund, between 8 and 26 weeks after the appearance of the tumor in the first 5 generations (average: 16.5 weeks), and between 4 and 11 weeks in the following generations (average: 8 weeks).

The implants grew evenly to a maximum size of 70 × 60 × 45 mm. They were usually well encapsulated and sometimes ulcerated. They were well vascularized and showed small areas of necrosis, generally in the center of the neoplastic mass. The consistency was always soft, the sectioned surface varied from reddish in the periphery to grayish in the center of the tumor, and there were some cavities containing hemorrhagic fluid. The transplanted tumors did not appear to invade the surrounding tissues. Metastases to the lung were observed in 23 animals (41.8%), to the inguinal and axillary lymph nodes in 2 animals (3.6%), and to the mesenteric lymph nodes, the spleen, and the kidneys in 1 animal each (1.8%).

Histologically, sections from the original tumors (fig. 7), showed a diffuse proliferation of oval-shaped and polyhedral cells with pale-to-clear cytoplasm and distinct cell borders. Interspersed between these cell masses there were numerous vascular channels, some of which were wide and conspicuous, while some were narrow and others had completely collapsed. All these vascular spaces appeared to have a distinct lining composed of spindle-shaped endothelial cells which were distinct and different from the proliferating tumor cells. The neoplastic cells showed pronounced mitotic activity. At the periphery there was invasion of skeletal muscle. There were several areas of necrosis and occasional focal deposits of calcific material. Sections stained with Snook's modification of the Maresch-Bielschowsky stain showed an abundance of argentaffine fibers which in most areas enveloped almost every cell (fig. 8). Particularly striking was the accentuation of the perivascular nature of the neoplastic proliferation by a more or less distinct argentaffine sheath around each vascular lumen separating the endothelial cells from the tumor cells (*37*). The basic pattern and cytology of the original tumor was well maintained in the transplants. There was greater variation in size and shape of the tumor cells, and mitotic activity was more pronounced than in the tumor of the original donor.

### Cutaneous Adnexal Cystadenocarcinoma, Resembling Hidradenoma (KR-2)

This tumor originated as a subcutaneous mass (3 × 5.5 cm) on the back of a 65-week-old female. This animal had received DMBA intratracheally for 59 weeks (200 μg in a gelatin suspension once a week). The tumor had been transplanted for 7 generations into 20 golden hamsters, 11 males and 9 females. It took in 17 animals (85%), and became palpable as a small nodule 1 to 8 weeks after transplantation (average latent period: 2.7 weeks).

The weight of the hosts bearing the transplants increased by 19.8 percent of the initial weight during the period of natural survival. The

tumor-bearing animals died or were killed when moribund, between 6 and 28 weeks (average: 12 weeks) after the appearance of the transplanted tumors, which grew evenly and constantly in all instances to a maximum of 50 × 40 × 15 mm. Sometimes ulcerated, they were slightly vascularized and showed a moderate amount of necrosis, predominantly in the center. On sectioned surface, they were usually cystic and varied from reddish to brown. The cysts contained hemorrhagic, mucoid fluid. Grossly, the tumors did not appear to invade the surrounding tissues. Pulmonary metastases were observed in 4 instances (20% of the tumor-bearing animals), and axillary lymph-node metastases in 1 (4%).

Microscopically the tumor of the donor showed a well-demarcated cystic structure (fig. 9). Numerous fibroepithelial formations composed of delicate branching strands of vascular connective tissue lined with cuboidal-to-tall columnar cells projected into the cyst. Many epithelial cells were distended and the usually eosinophilic cytoplasm was replaced by secretory globules, some of which were in the process of being discharged into the lumen. There were occasional well-circumscribed areas in which the tumor cells showed a moderate degree of atypia with deeper staining of the cytoplasm and moderate variation in size and shape of the nuclei. Occasional ductlike structures were seen in the capsule, but definite evidence of invasion was not observed. In some areas the tumor cells had a pale cytoplasm with distinct cell borders. Mitoses were infrequent. A lymph node near the tumor showed no evidence of invasion.

In the transplants, the tumor cells and papillary formations of the original tumor were duplicated (fig. 10) in some areas, while in others the growth appeared to be more solid. In the latter areas there were many clusters of large cells with distinct cell borders and pale or clear cytoplasm resembling the clear cells of hidradenoma. Often the centers of these clusters were occupied by a lumen containing pink-staining secretions. These groups of tumor cells were separated by delicate branching strands of a vascular fibrous stroma. In subsequent transplants the tumor maintained its pattern of cystic and solid growth, with many actively secreting glandular formations.

### Fibromyxosarcoma (LN-3)

The neoplasm originated in the right cheek pouch of a 49-week-old female hamster that had received a powdered diet containing 10 percent corn oil. This was one of the control animals in an experiment in which corn oil was the vehicle for the administration of a carcinogen. The tumor measured 2 × 2 × 1.5 cm and appeared to be encapsulated; it was soft and yellowish red. It was transplanted for 6 generations into 26 golden hamsters, 15 males and 11 females; it took in 22 animals (84.6% of recipients) and became palpable as a small nodule 3 to 13 weeks after transplantation (average latent period: 6 weeks). The weight of the hosts bearing the transplants increased on the average by 55 percent of

the initial weight during the period of natural survival. The tumor-bearing animals died or were killed when moribund, between 4 and 11 weeks after the appearance of the tumor (average: 10 weeks).

The transplanted tumors grew evenly and constantly to a maximum size of 70 × 50 × 20 mm. They appeared lobulated and slightly vascularized and showed a small amount of necrosis. The consistency was always soft, and the sectioned surface varied from pale yellow to reddish brown and was mucoid in appearance. The tumors did not invade the surrounding tissues and metastases were not observed.

Microscopically the central portions of the tumor were composed of bipolar and stellate cells with acidophilic cytoplasm and anastomosing cytoplasmic processes (fig. 11). These cells were separated by an abundant, faintly staining basophilic material. The tumor tissue formed globular masses projecting into a central lumen and contained many dilated and congested blood vessels. At the periphery the tumor had a more cellular appearance and was composed of spindle-shaped cells with elongated, fusiform nuclei. These cells resembled fibroblasts and infiltrated the surrounding muscle. Throughout the tumor there was a pronounced inflammatory reaction composed of lymphocytes and polymorphonuclear leukocytes. There were a few areas of necrosis.

In the transplants the predominantly myxomatous structure of the tumor was maintained (fig. 12). The neoplastic cells resembled those in the original tumor. There was a more pronounced degree of anaplasia with many atypical tumorous giant cells scattered throughout.

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PLATE 186

FIGURE 1.—Adenocarinoma of the breast. Original tumor. Hematoxylin and eosin. × 80

FIGURE 2.—Bile-duct carcinoma, intrahepatic (cholangiocarcinoma). Original tumor. Hematoxylin and eosin. × 100

FIGURE 3.—Cellular blue nevus-like melanotic tumor. First transplant. *Note* many elongated dendritic processes of neoplastic melanocytes. Hematoxylin and eosin. × 350

FIGURE 4.—Plasmacytoma. Origial tumor. A binucleated neoplastic plasma cell is seen in *center* of illustration. Hematoxylin and eosin. × 320

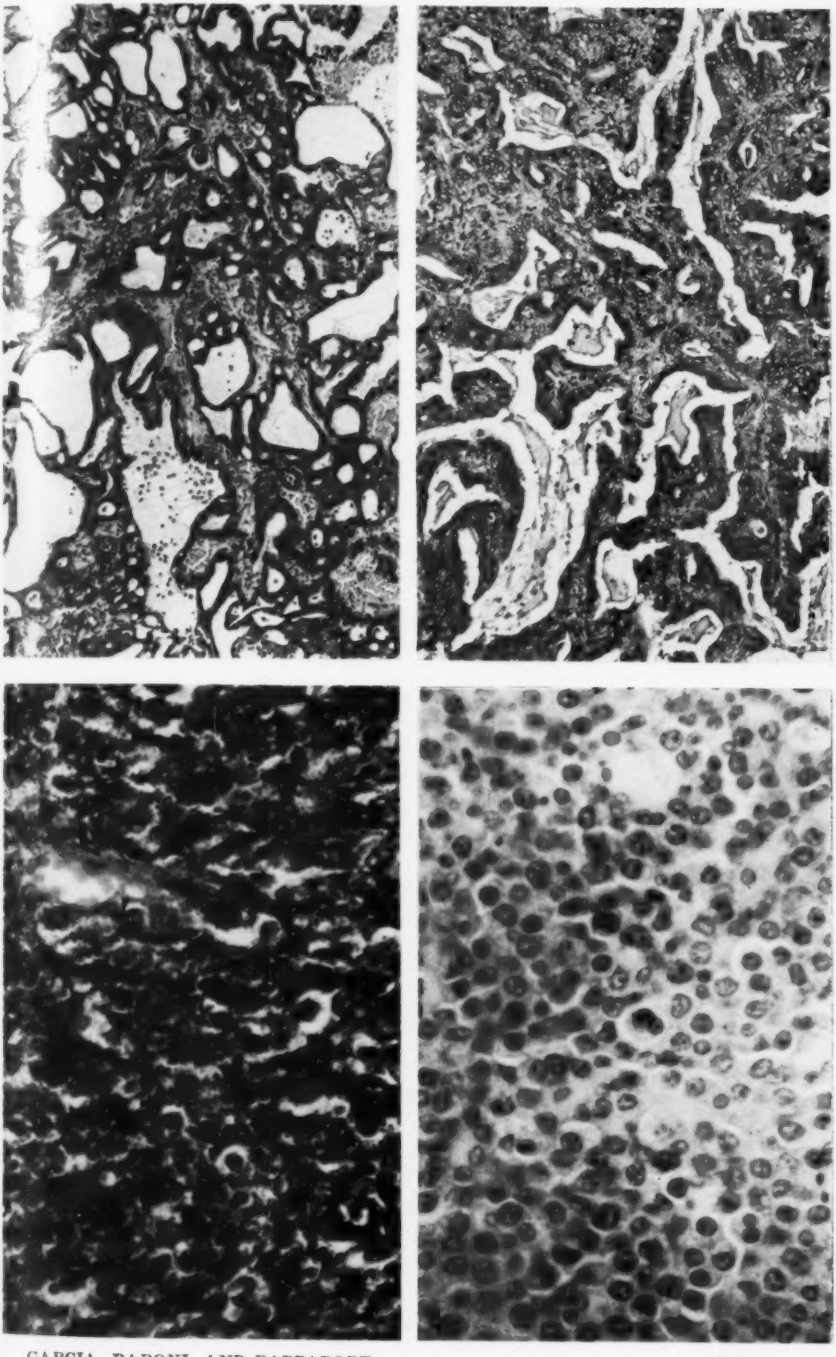

PLATE 187

FIGURE 5.—Malignant neurilemmoma. Original tumor. *Note* palisade-like arrangement of tumor cells. Hematoxylin and eosin. × 80

FIGURE 6.—Malignant neurilemmoma. Original tumor. In this area cell arrangement closely resembles that of Verocay bodies. Hematoxylin and eosin. × 225

FIGURE 7.—Hemangiopericytoma. Original tumor. *Note* extravascular proliferation of neoplastic cells. Hematoxylin and eosin. × 80

FIGURE 8.—Hemangiopericytoma. Original tumor. A delicate argentaffine membrane separates endothelial cells from extravascular proliferation of tumor cells. Silver impregnation for reticulin fibers. × 250

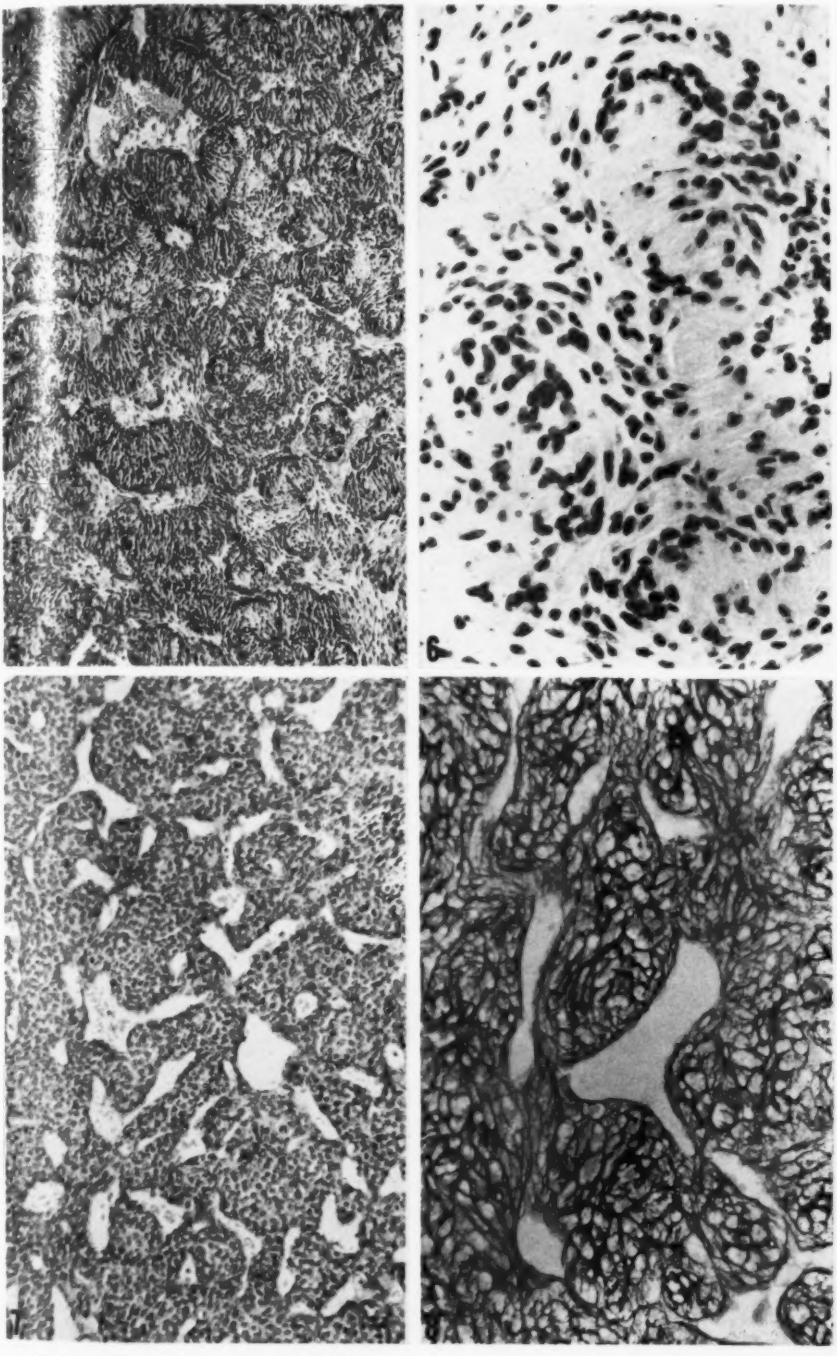



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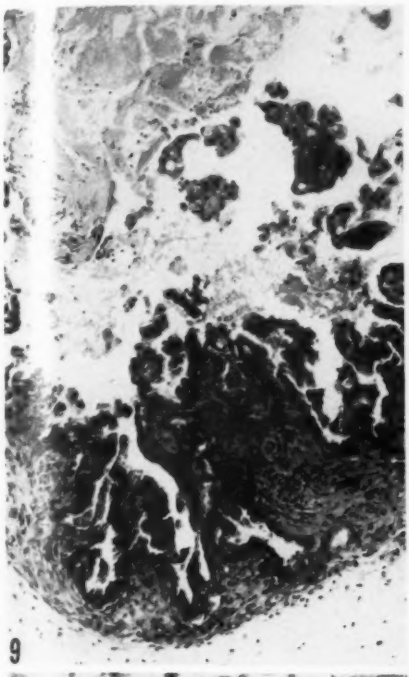
PLATE 188

FIGURE 9.—Cutaneous adnexal cystadenocarcinoma. Cystic portion of original tumor. Hematoxylin and eosin. × 100

FIGURE 10.—Cutaneous adnexal cystadenocarcinoma. First transplant. The papillary growth pattern is maintained in transplant. *Note* relatively pale cytoplasm and distinct cell borders of some cells and evidence of secretory activity. Hematoxylin and eosin. × 100

FIGURE 11.—Fibromyxosarcoma. Original tumor. Hematoxylin and eosin. × 225

FIGURE 12.—Fibromyxosarcoma. First transplant. Hematoxylin and eosin. × 225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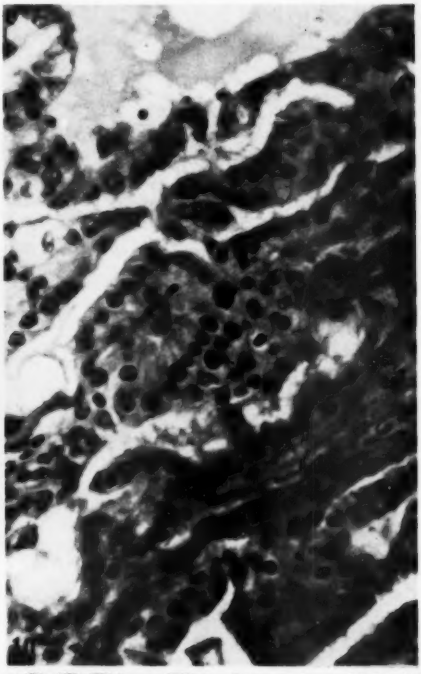

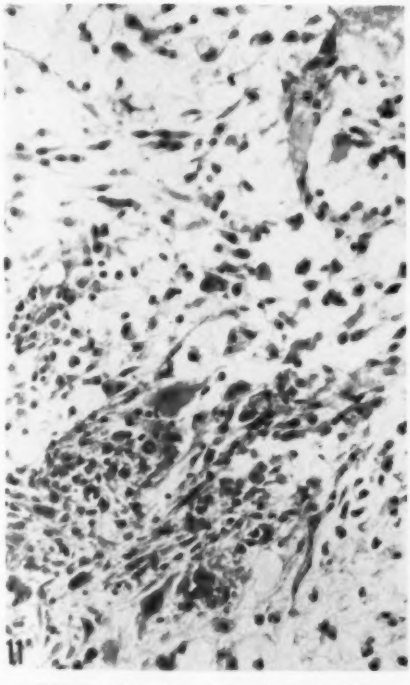
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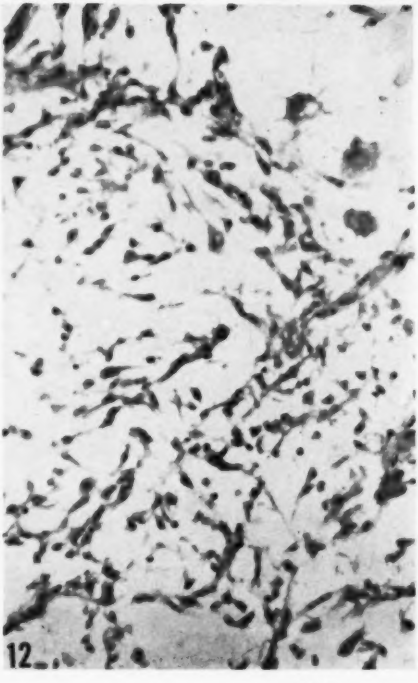
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# Biochemical and Morphologic Heterogeneity in a Transplantable Mast-Cell Neoplasm [1]

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SUMMARY

**The mast-cell neoplasm P-815 has been carried in its ascitic form for 180 transfer generations. In early generations, cells of this neoplasm contained numerous metachromatic granules and large quantities of serotonin and histamine. With repeated transfer, the levels of these metabolites remained relatively stable, but the number of cells with metachromatically staining granules decreased. With repeated transfer, some cells contained large intracytoplasmic vesicles, many of which were filled with eosinophilic material. A subline of this neoplasm was developed from an estimated 5 stemline cells. In the early generations of the subline, neoplasm serotonin content decreased and histamine content increased. Light and electron microscopic study revealed that the number of cells with vesicles increased sevenfold and those with granules decreased. With repeated transfer, the subline neoplasm returned to the biochemical and morphologic characteristics of the stemline, although its neoplastic virulence was permanently altered. These observations indicated biochemical and morphologic heterogeneity within this neoplasm.—J. Nat. Cancer Inst. 27: 1341–1359, 1961.**

THE MAST-CELL neoplasm P-815 arose in the subcutaneous tissue of a 16-month-old male DBA/2 mouse painted with methylcholanthrene. The original neoplasm was transplanted subcutaneously, and a stable ascitic neoplasm was developed from the solid tumor at the 3d transfer generation (*1*). The solid form of this neoplasm has been carried at the National Cancer Institute in strain DBA/2 mice for 120 transfer generations by Dr. Thelma Dunn, while the ascitic form has been carried in (BALB/c × DBA/2)$F_1$ hybrid mice for 180 generations by Dr. Michael Potter. In early generations both forms of the tumor contained numerous dense, metachromatic granules and large quantities of serotonin (5HT), histamine, and heparin (*1–5*).

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With repeated transfer, the solid tumor remained stable morphologically but lost almost all its 5HT content (80–180γ/g to 0–2γ/g), while histamine levels rose (470–560γ/g to 800–1200γ/g). In the ascitic form of P–815, the 5HT and histamine content has remained relatively stable, but the number of metachromatic-staining cells has decreased. For the present study, a subline of the ascitic mast-cell neoplasm was developed from a small number of P-815 cells (*6*), and the biochemical and morphologic characteristics of the 2 ascitic neoplasms were studied.

## MATERIALS AND METHODS

*Tumor systems and animals.*—The stemline ascitic mast-cell neoplasm was obtained from Dr. Potter at the 163d generation. It was maintained in our laboratory by weekly inoculation of 0.1 ml of a 1:10 dilution of ascites in Locke's solution into (BALB/c × DBA/2)$F_1$ hybrid mice. This neoplasm is referred to in the text as P-815A. A subline of this neoplasm was established at the 163d transfer generation by the inoculation of several mice with an estimated 5 cells from P-815A (*6*). The subline was initiated in one of the mice that developed ascites. This neoplasm is designated in the text as P-815M1. Transfer of this tumor has been performed as described for P-815A.

All mice in these experiments were kept in plastic cages, 5 mice to a cage, and were fed Purina chow pellets and tap water *ad libitum*. The $F_1$ hybrids were obtained from the Animal Production Unit, Laboratory Aids Division, National Institutes of Health.

*Histologic preparation.*—Fresh, thinly spread, air-dried smears of ascites were colored with Wright's stain in routine fashion and with toluidine blue by the method of Padawer (*7*).

*Electron microscopy.*—Ascitic fluid obtained by aspiration paracentesis was placed immediately into chilled chrome-osmium fixative (*8*) for 1 hour, hardened in 10 percent neutral isotonic formol for 1 hour, rapidly dehydrated with graded alcohol solutions, and embedded in 3 to 1 butyl and methyl methacrylates at 47° C with azo-diisobutyronitrile (ADIB) as a catalyst. Sections were cut on a Porter-Blum microtome with a diamond knife, mounted on carbon-coated copper grids, and stained with lead acetate and ammonium hydroxide vapors (*9*). Sections were then examined and micrographed with an RCA EMU 2c electron microscope.

*Assay of 5HT and histamine.*—Ascites was obtained by aspiration paracentesis, and aliquots of whole ascites varying from 0.05 to 0.5 ml were used. The volume of packed cells was determined in capillary microhematocrit tubes. After 3 washes in ice-cold Locke's solution, the cells were vibrated with a Raytheon Sonic Oscillator at 9 kc per second for 3 minutes in 5 ml of 0.4 N perchloric acid diluted to 8 ml with normal saline, and centrifuged for 30 minutes at 2500 rpm.

One ml of the supernatant was used for the determination of 5HT, according to the method of Weissbach *et al.* (*10*), and 4 ml of the super-

natant for the determination of histamine, by the method of Shore *et al.* (*11*). After the extraction of histamine, the method was modified because of the very high concentration of histamine in these cells. For fluorometric assay, 0.025 ml of the acid extract containing the histamine was added to 2 ml of 0.1 N HCl and the fluorophore with orthophthalaldehyde (OPT) developed as originally described.

Values for 5HT and histamine were expressed as micrograms per ml of packed cells.

## RESULTS

### Characterization of P-815A

*Biochemical determinations.*—The cells of current generations of the stemline neoplasm P-815A contained 200 to 260 $\gamma$ per ml packed cells of 5HT and 200 to 300 $\gamma$ per ml packed cells of histamine. These values were in the range of those noted in early generations (*5*). The 5HT and histamine content of unit volumes of packed cells of this neoplasm was remarkably constant from one generation to the next during routine transfer. During the first 5 days after tumor transplant, 5HT and histamine content slowly rose to levels previously indicated, but beyond the 5th day after transplantation, the cell content of these metabolites per unit volume of ascites was relatively constant. For this reason biochemical determinations were performed routinely on the 6th day after transfer. When the cells were harvested, the acellular portion of the ascites contained only 1 to 2 $\gamma$ per ml of 5HT and 5 to 6 $\gamma$ per ml of histamine.

*Cytologic studies.*—The stemline neoplasm P-815A has maintained most of the morphologic features originally described; however, 3 changes have been noted. There has been an increase in the basophilia (Wright's stain) of the cytoplasm. The number of cells with granules has decreased. While about half the cells contained granules that stained with Wright's stain, only about 5 percent of the cells contained granules that stained metachromatically with toluidine blue (fig. 2). Furthermore, the metachromatic granules, when present, were often smaller than in early generations. In recent generations of P-815A, 5 to 10 percent of the cells contained vesicles 2 to 5 $\mu$ in diameter, some of which were filled with eosinophilic staining material. These vesicles were not present in early generations of this neoplasm in either the ascitic or solid form. The remaining cells in the P-815A population contained neither granules nor vesicles (fig. 1). By light microscopic study it was thus possible to define 3 major cell types: those with granules, those with large vesicles, and those with neither granules nor vesicles. Mitotic figures were noted in all cell types.

Electron microscopic study of sectioned P-815A cells confirmed the morphologic heterogeneity noted with the light microscope in smear preparations, and the cell types previously described were identified. Certain structural characteristics were common to all cells. A distinct plasma membrane surrounded all cells. Small, finger-like cytoplasmic

processes of variable size, shape, and frequency, enclosed by the plasma membrane, projected from the periphery of the cells (figs. 5, 6, 7, 9, and 11). The nuclear profiles varied in size and shape in sectioned material and were spherical, elongated, kidney-shaped, or bilobed. Nuclear fine structure was consistent with previous descriptions, and a double nuclear membrane limited the nuclear material from cytoplasm. Mitochondria were abundant in all tumor cells and were easily identified. A well-developed Golgi region was identified in the juxtanuclear cytoplasm in most sections. The endoplasmic reticulum was poorly developed and showed little structural organization. However, a few strands of the ergastoplasmic portion of the endoplasmic reticulum were seen in the cytoplasm of most cells (figs. 5, and 7 through 11), and ribosomes unassociated with membranous structures were abundant throughout the cytoplasmic matrix. Small, clear vesicles up to 0.3 $\mu$ in diameter were also in the cytoplasm and were usually most numerous in the cell periphery just inside the plasma membrane (figs. 5 and 7).

As with light microscopy, it was possible to classify cells into 3 major groups.

The first of these was composed of cells that contained neither the large intracytoplasmic granules nor the huge intracytoplasmic vesicles (fig. 5). Some of these cells contained vesicular structures up to 1 $\mu$ in diameter within their cytoplasm (fig. 6). These vesicles were much smaller than, but similar in their fine structure to, the huge intracytoplasmic vesicles to be described that characterized another category of cells. These cells accounted for approximately 70 percent of sectioned cell profiles.

Cells in the second group were characterized by the presence of large intracytoplasmic granules varying from approximately 0.5 to 1.5 $\mu$ in diameter as seen in sectioned material (figs. 7 and 8). These granules were surrounded by a fine, electron-dense limiting membrane. Their internal contents varied in electron density and fine structure. The lighter, more electron-lucent granules were generally well preserved and filled with finely granular, homogeneous material (fig. 7). However, within some of these lighter granules, small circular islands of material of greater electron density were seen (fig. 8). The contents of the darker, more electron-dense granules usually revealed extensive fragmentation (figs. 7 and 8). Approximately 30 percent of sectioned profiles revealed the large, intracytoplasmic granules.

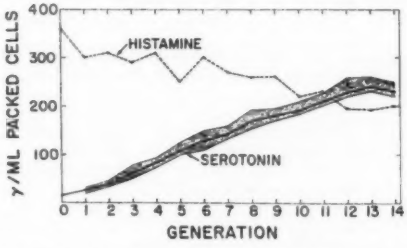
The third type of cell was characterized by the presence of huge intracytoplasmic vesicles 2 to 5 $\mu$ in diameter (figs. 9 through 12). The vesicles were limited from the surrounding cytoplasm by a fine electron-dense membrane. The internal structure and content varied considerably. Some contained sparse amounts of finely precipitated granular material within their limiting membrane (fig. 12); others had heterogeneous membranous structures suggestive of degenerating cellular debris (fig. 10). Still others contained the finely precipitated granular material, some material suggestive of cellular remnants, and also small vesicular structures 200 to 400 A in diameter (figs. 9 and 11). These cells were rarely

seen in P-815A and accounted for less than 5 percent of sectioned profiles of cells in that population.

No cells containing both large, intracytoplasmic granules and large vesicles were seen by electron microscopy, though they were seen rarely by light microscopy. It must be emphasized that some of the cells failing to show any large granules or vesicles in a single, thin section may have contained granules or vesicles in other portions of their cytoplasm not represented by that particular section. No definite virus-like particles were seen in any of the P-815A cells.

## Characterization of P-815M1

*Biochemical determinations.*—The 5HT content of the P-815M1 neoplasm in its first growth generation, when ascites accumulation was maximal, was much lower than that of the stemline P-815A, while the histamine content was higher. The cells of the subline neoplasm contained 15 γ per ml packed cells of 5HT and 340 γ per ml packed cells of histamine. During the succeeding 12 to 14 transfer generations, the 5HT and histamine content gradually returned to stemline levels. These changes are indicated in text-figure 1.




TEXT-FIGURE 1.—5HT and histamine content of P-815M1 neoplasm in 14 successive generations after its initiation. *Dot* = Average 5HT content of tumors from 5 mice in each generation; *stippled area* = range; *broken line* = average histamine content of tumors from 5 mice in each generation.

*Cytologic studies.*—Concomitant with the low 5HT and elevated histamine content in the early generations of P-815M1, definite morphologic differences from P-815A were evident in the tumor-cell population. By light microscopy the striking morphologic change was that the number of cells with vesicles had increased to account for 75 to 90 percent of the total cell population, the vesicles when present appeared larger, and the number of vesicles per cell increased. Fewer cells in this tumor contained metachromatic granules. A Wright-stained smear of the tumor with low 5HT content is shown in figures 3 and 4.

Electron microscopy of sectional cells of P-815M1 also revealed the change in the percentages of the 3 cell types previously described in the section dealing with the morphology of P-815A. Approximately 60 percent of the cell sections contained huge, intracytoplasmic vesicles (figs.

9 through 12), only about 5 percent contained the large, intracytoplasmic granules commonly seen in P-815A tumor cells, while the remaining cell profiles contained neither large vesicles nor granules. Again, no virus-like particles were seen. Except for this striking increase in the number of cells with huge intracytoplasmic vesicles and the decrease in the number of cells containing intracytoplasmic granules, no consistent morphologic differences between P-815M1 and P-815A tumors could be detected.

As the tumor-cell content of 5HT and histamine in P-815M1 returned to P-815A levels, light and electron microscopic study revealed a decrease in the number of vesiculated cells and an increase in the number of cells with granules, which thus showed a concomitant return of biochemical and morphologic characteristics to those of the stemline neoplasm.

*Neoplastic virulence.*—Tumor growth rate as evidenced by animal weight gain, increasing total ascites volume, and rising tumor cytocrit appeared to be the same in P–815A and P–815M1. However, the mean survival time of mice bearing the P–815A was 12 days, while the mean survival time of mice bearing P–815M1 was 10 days. This significant decrease ($P<0.01$) in survival time was in all generations of the subline neoplasm and has persisted to the present time (36 generations).

Table 1 summarizes the changes that occurred in early generations of the subline neoplasm P–815M1, as compared to the parental line P–815A.

TABLE 1.—Summary of alterations in early generations of subline neoplasm P–815M1 as related to parental line P-815A

| | |
|---|---|
| Serotonin content | Decreased |
| Histamine content | Increased |
| Cells with granules | Decreased |
| Cells with vesicles | Increased |
| Survival of mice bearing the tumor | Shortened* |

*Permanent change.

## DISCUSSION

The different cell types seen by light microscopy in the ascitic form of the stemline P–815A mast-cell tumor and the morphologic changes noted with repeated transfer demonstrated a morphologic heterogeneity within the tumor-cell population. This could have been due to a pre-established population variation or the occurrence of mutations over repeated generation transfers.

The fact that 5HT and histamine content of the tumor cells had remained relatively stable during repeated transfer suggested that the stemline neoplasm was in effect a balanced cell population. With the development of the subline neoplasm from an estimated 5 stemline cells, the balance of the cell population was disrupted with the resultant biochemical and morphologic alterations. The changes noted involved the

marked fall in 5HT content of tumor cells and striking increase in the number of cells in the new population that contained vesicles. In addition, histamine content had risen and the number of cells with metachromatically staining granules had decreased. However, the disruption of the cell-population balance was temporary, at least with respect to the morphologic and biochemical alterations noted, since during the first 14 transfer generations the subline neoplasm returned to stemline characteristics, which it has maintained to the present time (36 generations). This reversion suggested that a balance in the neoplastic-cell population had been re-established. The re-establishment of balanced cell populations in the P–815A neoplasm when 6-diazo-5-oxo-L-norleucine (DON) resistant and sensitive P–815 cells were mixed in varying proportions in tumor transfer inoculums has been noted by Potter (*12*). On the other hand, the persistent significantly shorter survival of mice bearing P–815M1 indicated that this subline neoplasm had acquired at least one lasting difference.

Schindler *et al.* (*4*) using an *in vitro* cloning method succeeded in developing sublines of P–815A that contained greater quantities of both 5HT and histamine than the stemline neoplasm. Since these lines were initiated from single cells, it may be assumed that these cells carried the potential to synthesize both 5HT and histamine. The percent increase in 5HT content in the subline was almost twice that of the histamine increase. The data indicated heterogeneity within the stemline-cell population, though detailed morphologic observations were not reported. Furthermore, the cell content of 5HT and histamine, the capacity to synthesize heparin, and the neoplastic virulence varied in cell lines derived from the parent neoplasm.

The data reported in the present study are in agreement with the observations in other mammalian tumors, which indicate the complexity of cell populations in tumor systems (*13–18*). Our findings add further emphasis to the concept that these neoplasms exist as multiclonal populations, with a wide range of biochemical, morphologic, and growth characteristics.

The question arises as to the origin of the variant cell types in this neoplasm. Whether they all exist prior to manipulative procedures or further changes occur in clones will be decided by long-term observations of sublines established from repeated cloning techniques that do not alter the growth characteristics of neoplastic cells. Undoubtedly, this tumor is cytogenetically unstable. This has been shown in this neoplasm (P-815A) by the work of Hauschka (*19*), the studies of Green and Day (*20*), and in other animal ascitic tumors by Levan (*21*). It is most likely that these changes occur as a result of the combined effects of physiological adaptions and single or successive mutational alterations. The differences in 5HT and histamine production noted in this study could be related to pre-established genetic variations in different cells with respect to ability to synthesize these metabolites. Additional support for this hypothesis may be found in the work of Green and Day (*20*) and Schindler *et al.*(*4*).

An alternate explanation might be that the cells were homogeneous in terms of potential to synthesize these metabolites, but that they varied in growth rates, with resultant secondary biochemical changes. This is less likely since Green and Day (*20*) showed that the amine levels of these cells in culture did not vary with the amount of cell inoculum, which, in that circumstance, determined generation time, *i.e.*, growth rate.

The association of the very low 5HT content of cells in P-815M1 with the decreased numbers of cells with granules was of interest, since previous work in another mast-cell tumor (*22*) showed a close relationship of 5HT and histamine to the large granule fraction of those cells. Furthermore, other work with P-815 indicated a closer association of heparin with 5HT than with histamine (*20*). Studies of the acid mucopolysaccharides in the cells of P-815M1 when the 5HT content was low would have been of interest, since it was possible that the altered 5HT content might have been secondary to altered mucopolysaccharide content with different 5HT and histamine-binding characteristics.

The fine structure of nontumor mast cells obtained from human tonsils (*23*) and the peritoneal cavities of mammalian laboratory animals (*24*), as well as tumor mast cells from dog and mouse mastocytomas (*25*, *26*) has been reported. All mast cells from these sources were described as containing large, intracytoplasmic granules. In contrast, many cells of P-815A and P-815M1 lacked these large, intracytoplasmic granules. Both P-815A and P-815M1 contained cells with huge, intracytoplasmic vesicles not previously described in either malignant or nonmalignant mast cells. No definite statement regarding the nature of these huge vesicles can be made. However, their internal structure suggests that phagocytosis by cells of ascitic fluid and cellular materials may contribute to their formation.

From the available data, no conclusions can be reached regarding the functional or biochemical significance of these structures. In this instance the observed alterations in morphology were paralleled by changes in the 5HT and histamine content of the tumor cells.

The morphologic alterations detected with the electron microscope were also seen with the light microscope, which indicates that, in this study, light microscopic examination of air-dried smears was an adequate technique for the detection of major morphologic alterations.

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# PLATES

PLATE 189

FIGURE 1.—Smear of P-815A ascitic mast-cell neoplasm. An occasional cell contains vesicles (*arrow*). Granules are present in some cells; others contain neither granules nor vesicles. Wright's stain. × 1,500

FIGURE 2.—Same as figure 1. Few cells contain metachromatic granules (*arrows*). Toluidine blue. × 750

FIGURE 3.—Smear of P-815M1 subline neoplasm. *Note* that many cells contain large cytoplasmic vesicles, some of which are filled with eosinophilic staining material. Wright's stain. × 1,500

FIGURE 4.—Same as figure 3. *Note* mitotic figure (*arrow*). Wright's stain. × 1,500

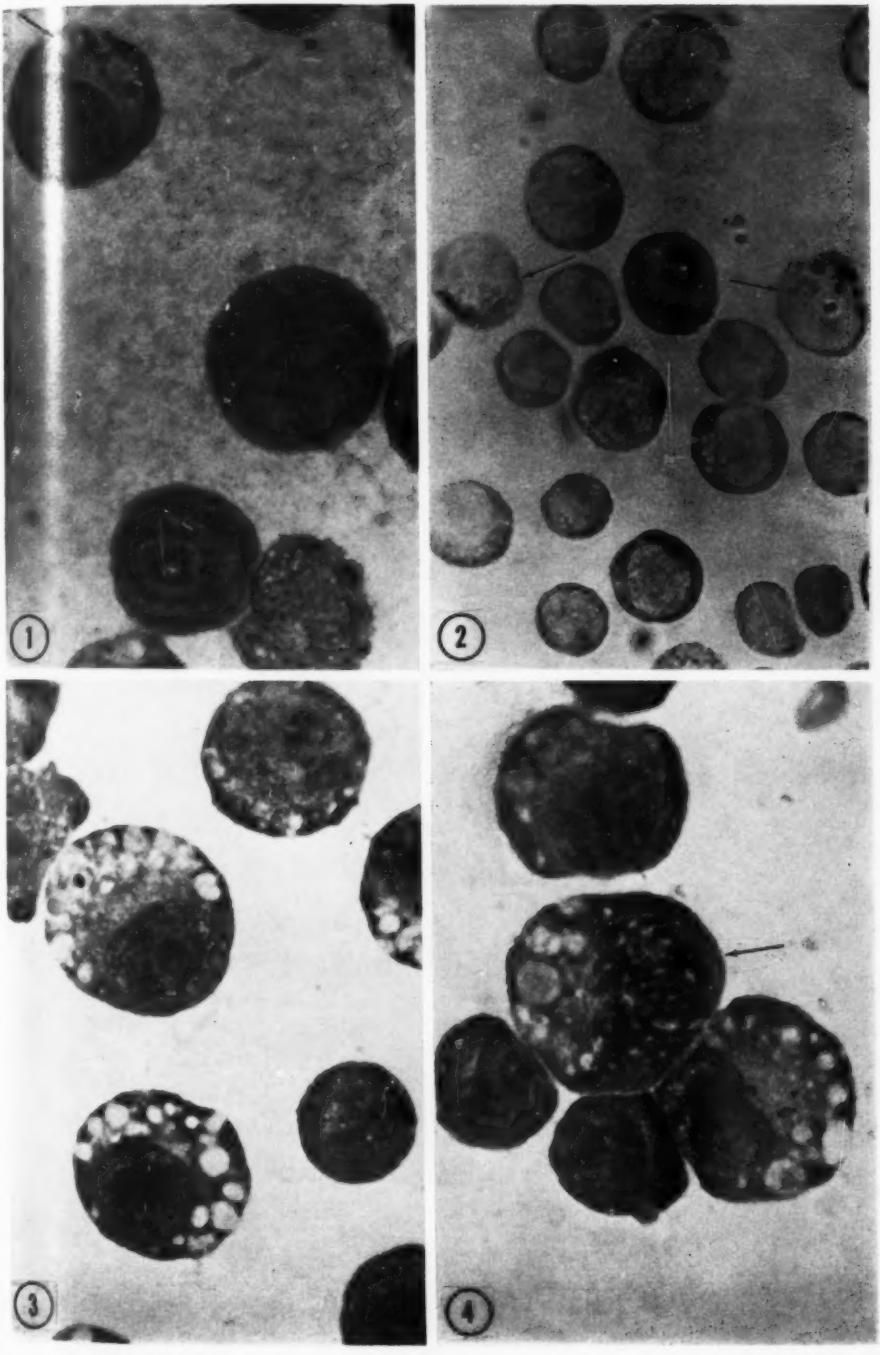




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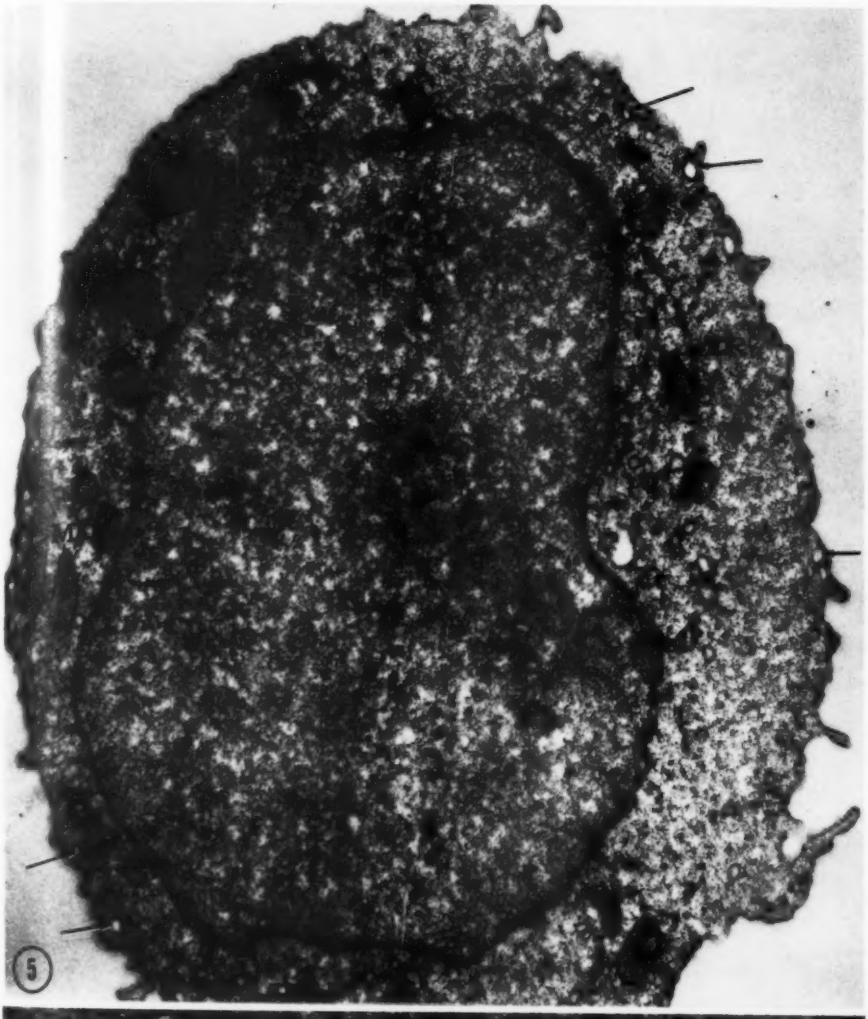
PLATE 190

FIGURE 5.—Section of a P-815A cell in which neither large intracytoplasmic granules nor large intracytoplasmic vesicles can be seen. Nucleus (N) occupies central portion of cell and is surrounded by cytoplasm. Many mitochondria (M) and a few strands of ergastoplasmic portion of endoplasmic reticulum (ER) are present in the cytoplasm. Many small submicroscopic vesicles (*arrows*) are seen in cytoplasm at periphery of cell. × 24,000

FIGURE 6.—Higher power electron micrograph of portion of cytoplasm of cell from P-815A containing neither large intracytoplasmic granules nor large intracytoplasmic vesicles. However, intracytoplasmic vesicles (V) up to 1 μ with internal structure similar to that seen in the large intracytoplasmic vesicles (*see* figs. 9, 10, and 11) are present. Golgi material (G) and mitochondria (M) can be identified in the cytoplasm. A small portion of the nucleus (N) is seen in *upper-left corner*. × 37,000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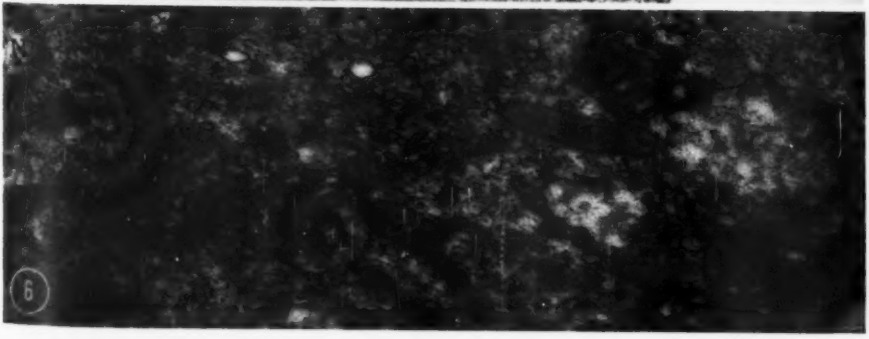
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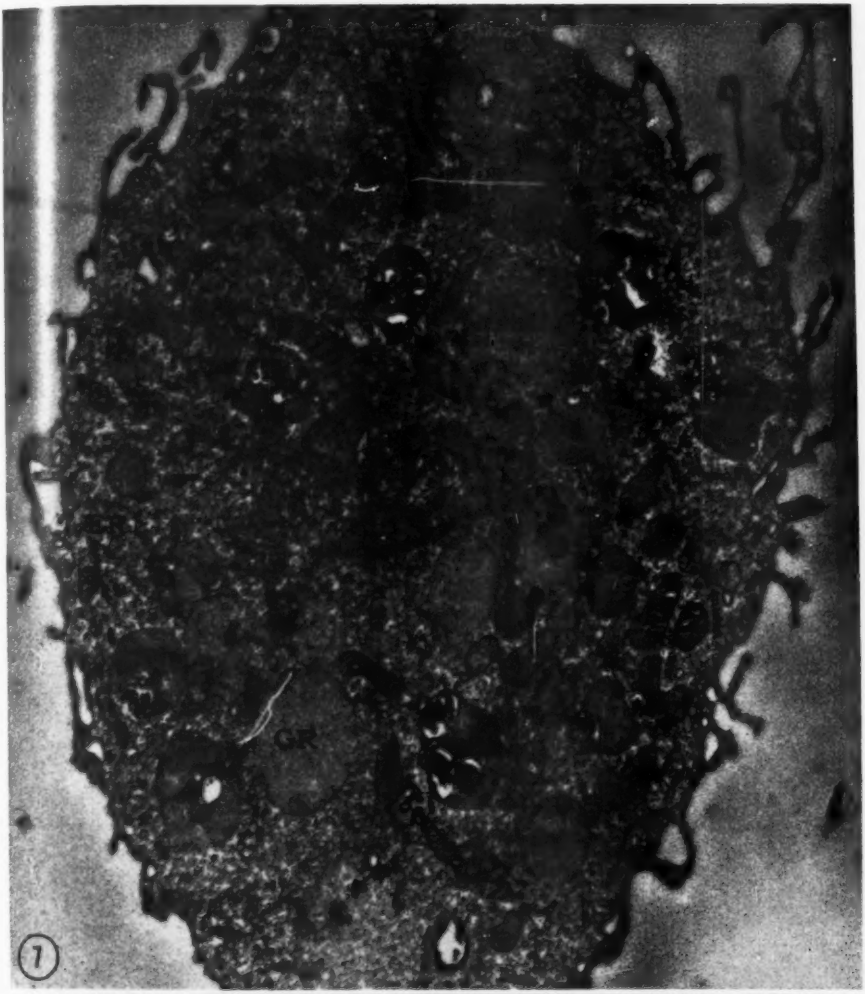
PLATE 191

FIGURE 7.—Section of cell from P–815A with many large intracytoplasmic granules (GR). The more electron-dense granules reveal some fragmentation of their internal structure. All granules are surrounded by a fine, dense membrane. Many mitochondria (M) and small strands of ergastoplasmic portion of endoplasmic reticulum (ER) are seen in cytoplasm. Portion of nucleus (N) is seen in center of cell. × 17,000

FIGURE 8.—Higher magnification of portion of cytoplasm of granular cell from P-815A. Both electron-dense and more electron-lucent granules (GR) are present. Small, round islands of electron-dense material (*arrows*) can be seen within lighter, more electron-lucent granules. × 30,000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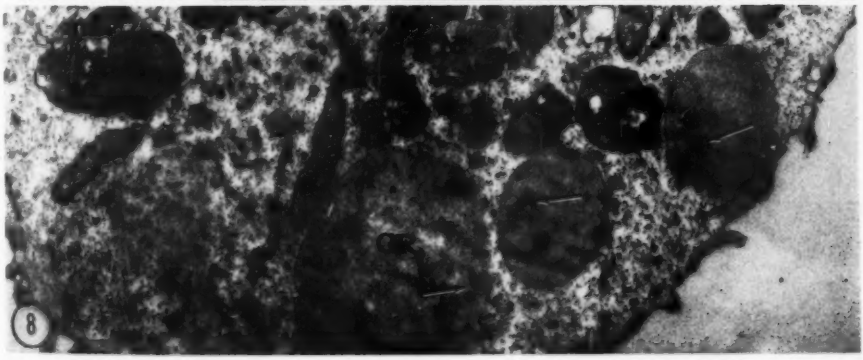
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PLATE 192

FIGURE 9.—Section of cell from P-815M1 containing huge intracytoplasmic vesicles (LV). Finely granular material is diffusely distributed within these vesicles. At periphery of some vesicles, very small vesicular profiles and larger globular, electron-dense structures (*arrows*) can be seen. Golgi material (G) can be seen in the juxtanuclear region of the cytoplasm. × 21,000

FIGURE 10.—Portion of cell from P-815M1 with large intracytoplasmic vesicle filled with heterogeneous material suggestive of cellular debris (CD). Small portion of the cell nucleus limited by characteristic double-layered nuclear membrane can be seen in the *lower-right corner*. × 33,000

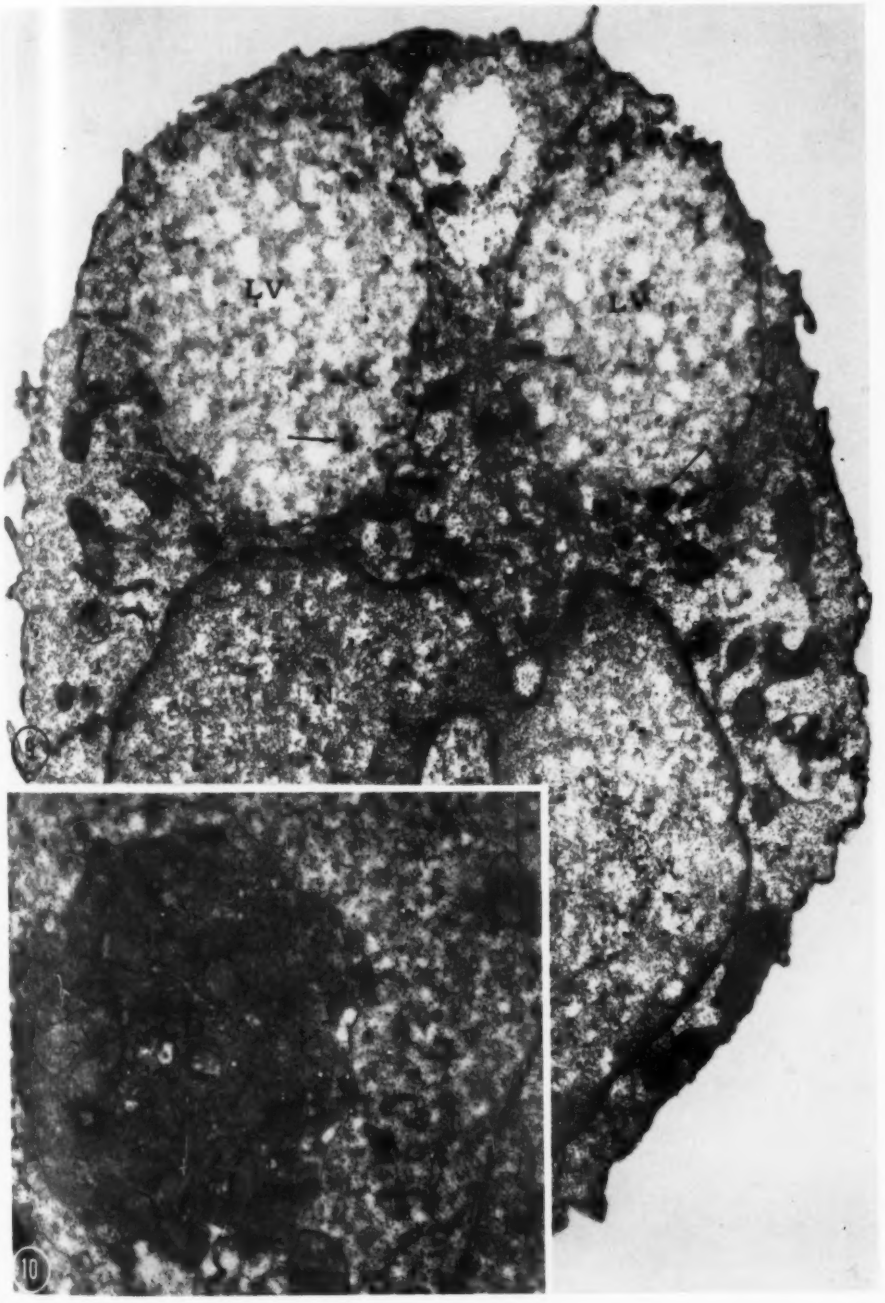
LV
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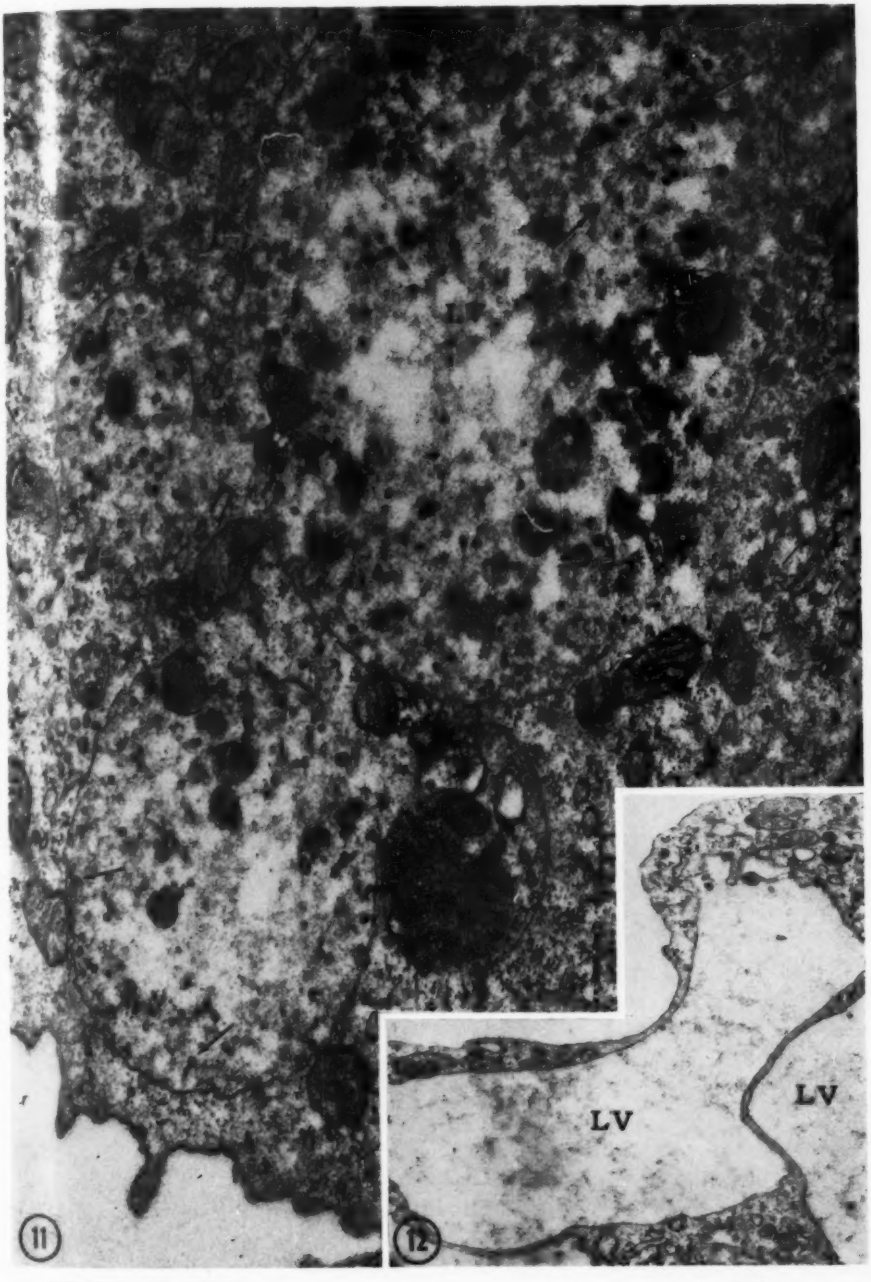
PLATE 193

FIGURE 11.—Portion of cytoplasm of cell from P-815M1 with large intracytoplasmic vesicles, showing in greater detail the variable internal structure of these large vesicles. Within the fine membranes enclosing vesicles finely granular material, small vesicular structures (*arrows*), and larger, electron-dense pleomorphic structures of various sizes can be seen. Ribosomes, mitochondria (M), and strands of ergastoplasmic endoplasmic reticulum (ER) can be identified in the cytoplasm surrounding the large vesicles. × 40,000

FIGURE 12.—Portion of cytoplasm of cell from P-815M1 with large intracytoplasmic vesicles (LV) containing only finely granular material. Thin strands of cytoplasm separate adjacent large vesicles. × 23,000



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# Failure of Bone Marrow to Interfere With the Augmentation of X-Ray Leukemogenesis by Urethan [1,2]

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SUMMARY

**Ten weekly intravenous injections of isologous bone marrow into C57BL/6 mice, each injection following an intraperitoneal injection of urethan, failed to inhibit the augmentation by the urethan of leukemogenesis induced by a previous, single, total-body X irradiation of 400 r. One intravenous injection of bone marrow depressed leukemogenesis by a single dose of 400 r X irradiation without any urethan treatment. These results do not support the possibility that the urethan effect on leukemogenesis causes a depression of the bone marrow, and thus lend further weight to the idea that its action represents a promoting factor, with irradiation serving essentially as initiating factor, in the relatively low dose ranges used. When bone marrow was injected soon after the irradiation (to inhibit leukemogenesis) *and urethan given subsequently*, the resulting leukemia incidence was considerably higher than that obtained with irradiation alone, and almost as high as with irradiation (without bone marrow) followed by urethan. It is suggested that the inhibiting effect of normal bone marrow on X-irradiation leukemogenesis probably operates at the initiating stage.—J. Nat. Cancer Inst. 27: 1361-1367, 1961.**

LEUKEMIA INDUCTION by total-body X irradiation, in strains of mice having a low spontaneous incidence of the disease, can be augmented by the simultaneous administration of urethan (ethyl carbamate) (*1*, *2*). The effect is also obtained when the urethan treatment is begun 2 weeks after the completion of the radiation treatment, but not when the procedure is carried out in reverse (*3*, *4*). The latter results are interpreted to mean that the urethan acts as a "promoting" factor and the irradiation, in relatively low dose ranges, as an "initiating" factor, according to the two-stage mechanism of carcinogenesis, as demonstrated in the skin (*5*, *6*).

Two other explanations had to be considered as possible alternatives to the two-stage mechanism: 1) urethan and X irradiation simply act

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[4] The authors wish to express their appreciation to Mr. Y. Serusi for valuable technical assistance, and to Dr. D. Davidov and the Tel Hashomer Hospital for facilities in irradiation of the animals.



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additively or synergistically, and 2) urethan causes a depression of the bone marrow over and above that resulting from total-body irradiation (*7–9*). The first alternative was made unlikely by the reversal experiment (*3, 4*) just quoted and by the observation that, under our experimental conditions, urethan alone seemed not to be leukemogenic. [From previous reports, urethan is nonleukemogenic in some strains (*2–4, 10, 11*) and leukemogenic in others, at least when administered soon after birth (*12, 13*).] The second alternative—the possible action of urethan by depression of the bone marrow—was thought to be improbable in view of the effectiveness of the urethan treatment as late as 2 weeks after completion of the radiation treatment (*8*).

However, more positive evidence was needed to exclude with certainty the indirect action of urethan via the bone marrow, since urethan is known to depress the mitotic activity of the bone marrow (*14, 15*). The present experiments were designed accordingly, by a repetition of the two-stage experiment for leukemogenesis with the addition of injections of normal bone marrow from isologous mice, at different stages in the experimental procedure, to determine whether such bone marrow injections could antagonize the urethan effect, as it is known to antagonize the leukemogenic action of total-body irradiation itself (*8*).

## MATERIALS AND METHODS

The mice used in these experiments were C57BL/6, originally obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and bred in this laboratory by brother × sister matings. They were randomized at the outset, included equal numbers of males and females in each group, and were 6 to 8 weeks old at the time of the first irradiation. The animals were housed in an air-conditioned room at 21 to 23° C, fed Purina laboratory chow, occasionally supplemented with barley and sunflower seeds, and provided with tap water *ad libitum*.[5]

The mice were irradiated in Lucite containers, 12 per group, each in a separate compartment, and received a single dose of 400 r total-body radiation. The physical conditions were: 230 kv, 15 ma, with 1 mm Al and 0.5 mm Cu filters; dose rate: 50 r per minute.

The bone marrow preparation was obtained from isologous C57BL/6 mice, 6 to 8 weeks old, by aspiration of the contents of the long bones (2 femurs and 2 tibiae from each animal) and suspended in Tyrode's solution, to provide a concentration of 60 to 80 × $10^6$ cells per ml. The suspension was injected into the tail vein, the number of cells ranging from 10 to 30 × $10^6$ per injection. In *groups 4 and 5* (*see* table 1), a single injection of the bone marrow suspension was given 20 to 24 hours after irradiation; in *group 2*, 10 weekly injections were given, each 24 hours after urethan treatment. The urethan solution—10 percent in

[5] As a routine precautionary measure, all the mice in this experiment were inoculated against ectromelia 3 weeks before the first irradiation, though there was no evidence of the disease in the laboratory after an epidemic occurring several years previously had been successfully eradicated, with a new colony of mice established.

sterile distilled water—was injected intraperitoneally, 0.2 ml (20 mg) administered once weekly for 10 weeks, totaling 200 mg of urethan per animal. With all injections, aseptic precautions were taken. The interval between irradiation and the first injection of urethan was 2 weeks.

All the surviving mice were killed 54 weeks after the first irradiation. These mice, and those dying during the experiment or which were obviously ill and had to be killed, were autopsied, and the following tissues kept for histological examination: thymus, spleen, liver, kidney, lung, and several lymph nodes, as well as any organ that appeared abnormal.

## RESULTS

Before we can analyze the results of the effect of bone marrow injections, attention should be drawn to the fact that, for simplification in the design of the experiments, the radiation was given as a single exposure of 400 r instead of 5 exposures of 90 r at intervals of 5 days, as in the previous studies (*3*, *4*). This accounts for the low incidence (7%) of induced leukemia by irradiation alone (*16*). That the experimental design was nevertheless effective for the purpose of the investigation is shown by the rise in incidence to 27 percent when the treatment was followed by urethan injections. The spontaneous incidence of leukemia in untreated mice of the same strain, age, and sex distribution, observed for the same period as for those under experiment, *i.e.*, till 62 weeks of age—corresponding to the age (6–8 weeks) at which the experimental animals were submitted to irradiation plus 54 weeks—the period of experiment and subsequent observation, was 0/141 mice.

The term "leukemia" is used here to comprise lymphomas, usually appearing first in the thymus but sometimes first in lymph nodes, occasionally displaying a lymphocytic leukemia blood picture, especially in later stages in the evolution of the disease, but also including a few cases with a generalized lymphomatosis or leukemia without involvement of the thymus. The pattern was thus similar to that observed by others in connection with X irradiation of C57BL mice (*16*, *17*). Because of the possibility that the generalized leukemia without thymus involvement may conceivably represent a spontaneous tendency not influenced by radiation treatment, table 1 also provides an analysis of the results in which the few nonthymic generalized leukemias are excluded.

The results of the effect of bone marrow may conveniently be considered in 3 parts (table 1):

*Groups 1 and 2* were designed to determine whether bone marrow injections *during the period of the urethan treatment* antagonized the augmenting effect of the latter with respect to the leukemogenic action of the initial total-body irradiation.

*Groups 3 and 4* were included to serve as a check for the efficacy of the bone marrow preparation, by tests of its activity when administered soon after total-body irradiation, without any urethan treatment.

TABLE 1.—Influence of bone marrow injections on X-ray leukemogenesis in C57BL/6 mice, with and without urethan treatment

| Group | Primary treatment* | Secondary treatment* | Number of mice used | Leukemia incidence† (%) | Leukemia incidence, excluding generalized nonthymic leukemia† (%) |
|---|---|---|---|---|---|
| 1 | Irradiation | Urethan | 85 | 20/72 = 27 | 19/72 = 26 |
| 2 | Irradiation | Urethan plus bone marrow | 85 | 18/72 = 25 | 18/72 = 25 |
| 3 | Irradiation | None | 97 | 7/97 = 7 | 6/97 = 6 |
| 4 | Irradiation plus bone marrow | None | 80 | 1/76 = 1 | 0/76 = 0 |
| 5 | Irradiation plus bone marrow | Urethan | 85 | 14/73 = 19 | 13/73 = 18 |

*Irradiation: single total-body exposure to 400 r. Urethan: 0.2 ml of 10 percent solution (20 mg) injected intraperitoneally and repeated at 10 weekly intervals (totaling 200 mg). A suspension of isologous bone marrow, injected intravenously once only in groups 4 and 5 (20–24 hours after irradiation), and repeated 10 times in group 2 (24 hours after each urethan injection). Interval between irradiation and first urethan injection was 2 weeks.

†Incidences of leukemia calculated on the basis of survivors at the time of the first appearance of leukemia in the whole series.

*Group 5* was included as an additional experiment to determine the effect of subsequent urethan treatment with respect to the prior depression of radiation leukemogenesis by bone marrow.

A comparison of the results of *groups 1 and 2* (table 1) shows that bone marrow injections during urethan treatment failed to inhibit the augmentation of radiation leukemogenesis by urethan. The leukemia incidence was 27 percent with total-body irradiation followed by urethan treatment, and 25 percent with the same treatment but including bone marrow injections following each urethan injection, *i.e.*, 10 weekly injections of bone marrow. The corrected figures (excluding generalized nonthymic leukemia) were 26 and 25 percent, respectively.

That these negative results are meaningful is shown from a comparison of *groups 3 and 4*, which demonstrates that even a single injection of the same preparation of bone marrow reduced the incidence of leukemia from 7 to 1 percent (corrected figures: 6 to 0%), when the bone marrow was injected shortly after the irradiation, without subsequent urethan treatment.

When bone marrow was injected soon after the irradiation, *i.e.*, as in *group 4*, but with urethan given subsequently, *group 5*, the resulting leukemia incidence was considerably higher than that with irradiation alone, though not quite as high as with irradiation followed by urethan, 19 percent, as compared with 7 and 27 percent, respectively (corrected figures: 18% as compared with 6 and 26%, respectively).

For completeness, these results are analyzed in table 2, in terms of individual weeks of death or killing of the animals in the various groups, both with and without leukemia.

TABLE 2.—Analysis of times of death in the various groups, counted from the time of the primary (X-irradiation) treatment

| Groups | Individual weeks of death or killing | | Number of survivors at end of experiment (54th week) | |
|---|---|---|---|---|
| | With leukemia* | Without leukemia*† | With leukemia | Without leukemia |
| 1 | 18, 19(2), 20, 21, 22, 23(2), 25(2), 26(2), 29, 30, 31, 32(2), 34, 44 | 19, 29, 30, 32, 34(2), 35, 37, 38, 39(2), 43, 48, 53(2) | 1 | 37 |
| 2 | 19, 23(3), 24, 26(3), 27, 29, 31, 32, 38, 39, 42, 45, 50 | 26, 30, 33, 39, 42, 45(2), 49(2), 51, 53(2) | 1 | 42 |
| 3 | 19, 22(2), 23, 27, 46, 49 | 19, 28(3), 39, 40(2), 41(2), 44(2), 45, 48(2), 50(3), 53 | 0 | 72 |
| 4 | 25 | 20, 24, 28, 30, 32, 33, 35, 36, 38, 39, 42, 45, 50(2) | 0 | 61 |
| 5 | 18, 20(2), 22, 23, 25, 26, 30, 38(2), 44, 47 | 21, 26(2), 28, 29(2), 30(2), 32, 34, 35, 36, 39, 40, 41, 42(2), 46, 47, 52, 53 | 2 | 38 |

*Figures in parentheses represent number of mice where more than 1 died in the same week.
†Recorded individual weeks of death without leukemia do not include deaths prior to the 18th week, the time of appearance of the first leukemia in the whole series.

## DISCUSSION

The primary outcome of these investigations was the demonstration that bone marrow injections failed to antagonize the augmenting effect of urethan on radiation leukemogenesis.

Since bone marrow injection after total-body irradiation is known to depress the leukemogenic action of the irradiation, as demonstrated by Kaplan and Brown (*8*) and others, and confirmed by the present work, our results may be interpreted to signify that the augmentation of radiation leukemogenesis by urethan is not due to a further depression of bone marrow activity, but operates in some other manner. This is all the more interesting in view of the fact that urethan is known to depress the mitotic activity of bone marrow (*14*, *15*).

In the failure to support the possibility of an indirect action of urethan via the bone marrow, added weight is given to the idea that the "co-leukemogenic" action of urethan represents a "promoting" effect as part of the two-stage mechanism. However, as previously pointed out (*3*, *4*), this is rather surprising, since in skin carcinogenesis urethan acts as a pure "initiator," with apparently no promoting action whatsoever (*18–22*).

Some indication of the role of urethan in leukemogenesis may be derived from the results of *group 5*, in which the urethan treatment was given after the leukemogenic action of irradiation had been depressed by bone marrow. Not only was the inhibiting effect of the bone marrow "re-

versed" by the subsequent urethan treatment, but the resulting leukemia incidence almost reached that obtained with irradiation followed by urethan.

This raises serious doubts whether the effect was indeed a "reversal" of bone marrow inhibition. When taken in conjunction with the results of *groups 1 and 2*, the evidence would suggest rather strongly that the action of urethan in relation to leukemogenesis is altogether unrelated to bone marrow activity, and that its site of action would have to be looked for elsewhere.

Regarding the interrelationship between the 3 factors discussed—X irradiation, bone marrow, and urethan—one might hazard the guess that the action of X irradiation, in the relatively low dose ranges used, is mainly at the initiating level; that bone marrow acts, in an inhibitory fashion, also at the initiating stage; and that urethan acts, in an augmenting fashion, at the promoting stage. But clearly, much more evidence would be needed before such a speculative concept could be accepted.

An attempt to correlate the results reported here with the known participation of a viral factor in leukemogenesis (*23*, *24*) would seem out of place in this discussion. It will, however, be dealt with in a separate communication (*25*).

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# Intracellular Localization of "Hematoporphyrin" in a Transplanted Tumor [1, 2]

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SUMMARY

**The intracellular localization of porphyrin taken up by a transplanted rat tumor has been studied. Quantitative determination of the porphyrin concentrations in the subcellular fractions and direct observation by fluorescence microscopy indicate that porphyrin accumulates in the soluble fraction and not in the cell wall, nucleus, mitochondrial, or microsomal particles. There is suggestive evidence that selective uptake by the soluble fraction is due to a specific constituent to which the porphyrin is bound.—J. Nat. Cancer Inst. 27: 1369–1377, 1961.**

IT HAS been reported that porphyrins accumulate in tumor tissue (*1–5*). The earliest studies described red fluorescence with the typical emission spectrum of porphyrins in human and experimental tumor specimens. Later work with animals demonstrated that parenterally administered porphyrins accumulate in embryonic, lymphatic, regenerating, and neoplastic tissues in rough proportion to their mitotic indexes (*6*, *7*).

The phenomenon of selective uptake in tumor tissue of parenterally administered porphyrin has not yet found successful application in clinical situations. It has failed as a guide to radical cancer surgery (*8*), as a means of tumor localization by scintiscanning for an incorporated radioactive metal (*9*), and was not consistently effective in the potentiation of radiotherapy on tumors (*10*).

Despite this background of clinical and laboratory observations, there are few studies that provide quantitative data regarding the phenomena. In a preceding paper by Winkelman and Rasmussen-Taxdal (*1*), the quantitative uptake of porphyrin in tumor was compared to that in control tissue, and preliminary observations on the transformations of the porphyrin in tumor tissue were reported. After administration of hematoporphyrin to rats bearing the Walker carcinosarcoma 256, more than 2 μg per g of porphyrin was found in the tumor tissue, and a negligible

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[2] This work was done in collaboration with the Laboratory of Technical Development, National Heart Institute, Bethesda, Md.

[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

amount in control muscle tissue. In this paper, a study of the intracellular localization of exogenous porphyrin in tumor cells is reported. Evidence is presented from both quantitative determination of porphyrin in the subcellular fractions and direct demonstration with fluorescence microscopy.

## MATERIALS AND METHODS

*Porphyrin.*—Hematoporphyrin hydrochloride (NENCKI), recrystallized, was obtained from the Cosmos International Corporation, New York, New York. This is the same material used in the previous study (*1*). It contains no detectable protoporphyrin or deuteroporphyrin and has the absorption spectrums and melting point of the dimethyl ester typical for hematoporphyrin.

*Animals and tumor.*—Female Sprague-Dawley rats weighing approximately 250 g were used in all experiments. Pellets of 1 $mm^3$ of Walker carcinosarcoma 256 were implanted subcutaneously in the flank of the rat through a trochar. Eleven days after tumor implantation, 30 mg of hematoporphyrin solution (10 mg per ml in physiological saline) was injected intraperitoneally. The animals were killed 24 hours after injection. There was no grossly visible necrosis or hemorrhage in the tumors studied.

*Fractionation.*—Tumor specimens were rapidly excised and placed in beakers containing ice-cold saline solution. The whole tumor was decapsulated, then minced and homogenized in 0.25 M sucrose (9:1, v/w) with a Potter-Elvejhem homogenizer. The homogenate was separated into nuclear, mitochondrial, microsomal, and supernatant fractions by differential centrifugation (*11*). This method has been applied by Busch *et al.* (*12*) to the Walker carcinosarcoma 256, and the fractions so obtained were studied microscopically. The 600 $\times$ *g* precipitate contained only nuclei. Despite some variability in the histologic components of the other fractions, they were treated as single fractions by Busch. In this study the original procedure (*11*) to obtain the soluble fraction was modified by centrifugation at 100,000 $\times$ *g* for 60 minutes instead of 30,000 $\times$ *g* for 60 minutes to remove ribosomal material that otherwise would have remained in the supernatant solution.

*Extraction and quantification.*—Porphyrin was extracted from the subcellular fractions by the method used previously to obtain total porphyrin from unfractionated homogenates (*1*, *13*).

1) The nuclear, mitochondrial, and microsomal fractions, obtained as pellets from the centrifuge tubes, were homogenized in 10 ml of buffered acetic acid (4 parts glacial acetic acid to 1 part saturated sodium acetate) and 30 ml of ethyl ether. The soluble fraction, obtained as the 0.25 M sucrose supernatant of the 100,000 $\times$ *g* centrifugation, was saturated with sodium acetate and diluted with 4 times its volume of acetic anhydride. Ethyl ether, 3 times the volume of the sodium acetate-acetic anhydride solution, was added. Then more acetic anhydride was added

until the 2 phases became miscible. Finally, the ratio of buffered acetic acid to ethyl ether was adjusted to 1:3 by the addition of ethyl ether.

2) The flocculent precipitate that formed in the buffered acetic acid-ethyl ether system was removed by centrifugation at 4000 rpm for 5 minutes. The supernatant solution was filtered into a suction flask through Whatman #1 filter paper.

3) The ether-acetic acid filtrate was washed 4 times with 10 ml 3 percent sodium acetate in a separatory funnel. The acetic acid-sodium acetate washes were checked for porphyrin by the absence of red fluorescence and discarded. Porphyrin added to the wash solution confirmed that fluorescence would have occurred if porphyrin had been present.

4) The ether was extracted 3 times with 5 ml of 5 percent HCl or until there was no red fluorescence in further HCl extractions. The combined 5 percent HCl extract was assayed for porphyrin content by fluorometric and spectrophotometric methods. Standards were hematoporphyrin in 5 percent HCl. For fluorometric analysis, the Aminco-Bowman spectrophotofluorometer was used. The optimal activating wavelength of 408 mμ gave maximum fluorescence emission at 600 mμ. If the slits and meter multiplier were set for maximum sensitivity, 0.01 μg hematoporphyrin per 100 ml 5 percent HCl could be determined. The spectrophotometric method measured the absorption in the Soret band, at 402 mμ, in the Beckman DU spectrophotometer.

*Fluorescence microscopy.*—Monolayers of dispersed individual cells and clumped cells from touch preparations of freshly cut surfaces of tumors were dried in air and mounted in saline. Their fluorescence was observed with a Leitz Ortholux microscope with standard accessories—ultraviolet light source from a high-pressure mercury arc lamp, Leitz UVt 2 mm UG 1 short-range ultraviolet transmitting filter, dark-field condenser, and an ultraviolet protective filter between the ocular and eyepiece. Fluorescence microphotographs were taken on Super Anscochrome Tungsten Type Film with a Leica Micro-Ibso attachment and Leica If camera.

## RESULTS AND DISCUSSION

Table 1 summarizes the data obtained by quantitative analysis of the subcellular fractions of Walker carcinosarcoma 256. Results of fluorometric and spectrophotometric analyses of each sample are in adjacent columns. The spectrophotometric method, which detects porphyrin and metalloporphyrin, usually gives values 2 or 3 times greater than the fluorometric method, which detects only porphyrin (*13*). Data from the first 7 animals establish control values for the endogenous porphyrin content of the subcellular fractions of tumors that had grown for 12 days. Data from the following 15 experimental animals give the total porphyrin content of the subcellular fraction of 12-day-old tumors, 24 hours after the intraperitoneal injection of 30 mg of hematoporphyrin. Mean values and standard deviations are given.

TABLE 1.—Porphyrin content of tumor subcellular fractions

| | Microgram porphyrin/g tissue, wet weight | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Nuclei | | Mitochondria | | Microsomes | | Soluble fraction | |
| Animal* | Fluorometric | Spectrophotometric | Fluorometric | Spectrophotometric | Fluorometric | Spectrophotometric | Fluorometric | Spectrophotometric |
| **Controls** | | | | | | | | |
| 1 | 0.05 | 0.11 | 0.27 | 0.37 | 0.085 | 0.21 | 0.04 | 0.10 |
| 2 | 0.04 | 0.23 | 0.18 | 0.35 | 0.04 | 0.07 | 0.06 | 0.09 |
| 3 | 0.09 | 0.18 | 0.12 | 0.29 | 0.12 | 0.16 | 0.06 | 0.08 |
| 4 | 0.20 | 0.64 | 0.18 | 0.29 | 0.11 | 0.14 | 0.02 | 0.04 |
| 5 | 0.06 | 0.21 | 0.18 | 0.37 | 0.04 | 0.09 | 0.04 | 0.07 |
| 6 | 0.20 | 0.49 | 0.12 | 0.30 | 0.07 | 0.19 | 0.04 | 0.09 |
| 7 | 0.13 | 0.23 | 0.19 | 0.21 | 0.06 | 0.10 | 0.03 | 0.05 |
| Mean ± SD | 0.11 ± 0.06 | 0.30 ± 0.17 | 0.18 ± 0.05 | 0.31 ± 0.04 | 0.08 ± 0.03 | 0.14 ± 0.05 | 0.04 ± 0.01 | 0.07 ± 0.02 |
| **Experimental†** | | | | | | | | |
| 1 | 0.16 | 0.24 | 0.465 | 0.72 | 0.19 | 0.41 | 2.65 | 5.38 |
| 2 | 0.28 | 0.42 | 0.22 | 0.43 | 0.11 | 0.36 | 0.93 | 1.98 |
| 3 | 0.11 | 0.26 | 0.19 | 0.41 | 0.06 | 0.14 | 0.39 | 1.08 |
| 4 | 0.16 | 0.34 | 0.07 | 0.20 | 0.04 | 0.11 | 0.47 | 1.31 |
| 5 | 0.65 | 1.15 | 0.31 | 0.77 | 0.14 | 0.18 | 0.98 | 2.40 |
| 6 | 0.16 | 0.33 | 0.12 | 0.31 | 0.15 | 0.30 | 0.68 | 1.95 |
| 7 | 0.31 | 0.98 | 0.15 | 0.30 | 0.30 | 0.44 | 0.45 | 1.65 |
| 8 | 0.43 | 0.92 | 0.12 | 0.24 | 0.08 | 0.11 | 0.56 | 1.46 |
| 9 | 0.42 | 0.91 | 0.15 | 0.23 | 0.16 | 0.21 | 1.08 | 2.15 |
| 10 | 0.26 | 0.55 | 0.19 | 0.51 | 0.25 | 0.53 | 0.79 | 1.13 |
| 11 | 0.27 | 0.68 | 0.15 | 0.42 | 0.14 | 0.36 | 1.22 | 3.15 |
| 12 | 0.43 | 0.89 | 0.14 | 0.38 | 0.09 | 0.19 | 1.19 | 4.57 |
| 13 | 0.32 | 0.32 | 0.08 | 0.28 | 0.10 | 0.23 | 1.06 | 1.62 |
| 14 | 0.19 | 0.47 | 0.51 | 1.01 | 0.10 | 0.57 | 1.93 | 3.52 |
| 15 | 0.58 | 0.79 | 0.59 | 1.83 | 0.25 | 0.42 | 2.08 | 3.20 |
| Mean ± SD | 0.32 ± 0.15 | 0.62 ± 0.29 | 0.21 ± 0.15 | 0.47 ± 0.24 | 0.14 ± 0.07 | 0.31 ± 0.14 | 1.10 ± 0.63 | 2.44 ± 1.24 |

*All animals had tumor growing for 12 days when killed.
†Experimental animals all received 30 mg hematoporphyrin on the 11th day of tumor growth, 24 hours before they were killed.

It can be seen by examination of table 1 that endogenous porphyrin is lowest in the soluble fraction of control tumor tissue. The mitochondria, in which the porphyrin-containing cytochromes are localized, possess the highest native concentration of porphyrin. However, the increase in porphyrin concentration of this fraction in the experimental tissue is less than twofold, the least of all the fractions. The increase in the porphyrin content of the soluble fraction is 30-fold, that of the nuclear and microsomal fractions two- or threefold. Both the total porphyrin content and the ratio of porphyrin in the experimental tissue to that in the control tissue are greatest in the soluble fraction.

The results are also presented in terms of porphyrin per mg of protein, to demonstrate that the localization of porphyrin in the soluble fraction is not merely a distribution within the various cell compartments proportional to their volumes, and that the porphyrin is not actually concentrated with respect to protein in some other fraction. Protein determinations (*14*) were made of the subcellular fractions of the 12-day-old tumor. The fractions were obtained from specimens other than those used for porphyrin analysis by the same method that Busch *et al.* (*12*) employed to evaluate the homogenization and differential centrifugation procedure for the Walker carcinosarcoma 256. The results are shown in table 2, with Busch's data presented for comparison. There is good agreement between the two sets of values. The "specific activity" of each fraction, defined as $\mu$g porphyrin per mg protein, is shown in table 3. The increase in "specific activity" from control to experimental is 2.1-fold for nuclei, 1.5-fold for mitochondria, 2.2-fold for microsomes, and 35.4-fold for the soluble fraction. Although the "specific activity" of the mitochondria is high, it is clearly in the soluble fraction that the increase of porphyrin concentration per unit weight of protein is greatest.

An unusual difficulty encountered in the extraction of porphyrin from the soluble fraction also supports the specificity of selective uptake of porphyrin by this fraction. Despite the fact that hematoporphyrin is completely extracted from neutral aqueous solutions by ether (*13*), the typical red fluorescence of porphyrin remained in the neutral 0.25 M sucrose soluble fraction after it was shaken with ether in a separatory funnel. The porphyrin was not extracted by a variety of rigorous treatments, including *p*H variation from 2 to 14, heating at 50° C for 10 minutes, and addition of dioxane, pyridine, acetone, methanol, or ethanol. The porphyrin was readily extracted with ether from 0.25 M sucrose solutions of porphyrin, 0.25 M sucrose suspensions of the other tumor-tissue fractions, and suspensions to which additional porphyrin was added. These observations strongly suggest the presence of a constituent of the soluble fraction of the tumor that binds porphyrin so tightly that it cannot be extracted by ether from neutral aqueous solutions.

The total uptake of porphyrin by the tumor found in the previous study (*1*) agrees reasonably well with the sum of the porphyrin contents of the different fractions in this study. However, the results of the two studies are not strictly comparable because of variation in the dose

TABLE 2.—Protein content of subcellular fractions of Walker carcinosarcoma 256 (mg/g wet weight)

| Tissue | Number of experiments | Whole homogenate | Nuclei | Mitochondria | Microsomes | Soluble fraction | Recovery (%) |
|---|---|---|---|---|---|---|---|
| 12-Day-old tumor | 6 | 128 (117–145)* | 56 (50–63) | 7.1 (6.5–9.1) | 11.1 (8.4–12.8) | 31 (27–34) | 82 |
| 7-Day-old tumor† | 3 | 111 (86–148) | 48 (37–66) | 5 (4–6) | 8 (5–10) | 29 (22–40) | 81 |

*Ranges are given in parentheses.
†Values of Busch *et al.* (*11*).

TABLE 3.—"Specific activity" of subcellular fractions (μg porphyrin/mg protein)*

| | Nuclei | Mitochondria | Microsomes | Soluble fraction |
|---|---|---|---|---|
| Control | 0. 0054 | 0. 044 | 0. 012 | 0. 0022 |
| Experimental | 0. 011 | 0. 066 | 0. 027 | 0. 0781 |
| Experimental / Control | 2. 1 | 1. 5 | 2. 2 | 35. 4 |

*The porphyrin concentrations used here were obtained by the spectrophotometric method.

of exogenous porphyrin, the interval between injection and killing, and the method of fluorometric analysis.

The quantitative evidence that porphyrin is localized in the soluble fraction is supported by the microscopic distribution of the red fluorescence. The accompanying plate shows a fluorescence photomicrograph of a touch preparation of individual and dispersed clumps of cells. The red fluorescence appears uniformly distributed in the cytoplasm of each cell when viewed under oil immersion. There are no apparent accumulations within the cytoplasm that suggest mitochondrial concentration (*15*), or in the discrete cytoplasmic and nuclear patterns such as Mędraś described for the endogenous porphyrin of chrysoidin-induced mouse hepatomas (*16*). Fluorescence is not concentrated at the cellular or nuclear membranes. The cell fractionation alone would not eliminate the possibility that during the homogenization or centrifugation procedure porphyrin entered the soluble fraction from some other fraction to which it had orginally belonged. The diffuse distribution of the fluorescence in the cytoplasm of the intact cells, however, makes this possibility unlikely.

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## PLATE 194

FIGURE 1.—Black-and-white reproduction of the fluorescence photomicrograph of touch preparation from Walker 256 carcinosarcoma. Oil immersion; 10-minute exposure. *White areas* represent red fluorescing areas in original color photograph. × 450

# Protective Effect of Parasympatheticomimetic Agents on Ciliated Mucus-Secreting Epithelium [1,2]

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SUMMARY

**Exposure of ciliated mucus-secreting epithelium of the esophagus of the frog to parasympatheticomimetic agents protects against the effects of irritant aerosols so that the slowing of mucous stream flow induced by the latter does not occur. The agents studied include 1) acetylcholine and choline-like drugs, 2) cholinesterase inhibitors, and 3) compounds related to nicotinic acid with parasympatheticomimetic properties. Prior application of parasympatheticomimetic agents exerts a prophylactic effect against the subsequent action of irritants. In both instances, protection persists for periods up to 6 hours. Ciliary activity and mucous secretion appear to be locally controlled by acetylcholine activity. Concentrations below those of local or systemic toxicity are capable of inducing the response. Irritants present in the particulate phase of cigarette smoke and urban polluted air induce a slowing in the rate of flow of the mucous stream, probably as a result of alteration of ciliary activity and modification of mucous secretion. *In vivo* this has been shown to facilitate prolonged retention of particulate matter settling on the respiratory epithelium of mice, rats, and rabbits. When the particulates consist of or contain known carcinogenic agents, their possible role in the pathogenesis of pulmonary cancer is suggested. The experimental and clinical implications of this finding are discussed.—J. Nat. Cancer Inst. 27: 1379–1392, 1961.**

IN EARLIER publications the effects of irritant aerosols in urban polluted air and cigarette smoke on the respiratory epithelium of several mammalian species and on the ciliated mucus-secreting epithelium of the esophagus of a frog were described (*1*, *2*). These effects are primarily characterized by a slowing of the flow of the mucous stream overlying the epithelium, and the decrease in rate apparently is the result of qualitative and quantitative alterations in the mucus and changes in the ciliary

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[3] The photographic work was done by Mr. Lloyd Matlovsky, Department of Photography of the University of Southern California School of Medicine.



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activity of the epithelium. A critical factor in the prevention of prolonged residence of particulate matter on the lining cells of the tracheobronchial tree is the cephalad flow of the mucous stream that covers the epithelium and carries particles to the pharynx where they are swallowed or otherwise removed. The studies of Proetz (*3*), Hilding (*4*, *5*), Dalhamn (*6*), as well as our own (*7*), suggest that interference with this defense mechanism may be a factor in the pathogenesis of pulmonary cancer.

Epidemiological studies have demonstrated a world-wide increase in the frequency of lung cancer (*8*, *9*). These investigations have indicated that urban polluted air and excessive cigarette smoking may have significant etiologic implications. Carcinogenic hydrocarbons have been demonstrated in the particulate phase of both environmental sources (*10*, *11*). These carcinogens occur in a size range consistent with the inhalation and deposition of the particles on the respiratory epithelium. Under physiologic conditions, the continuous flow of the mucous stream on the respiratory epithelium tends to preclude the accumulation and ultimate prolonged retention of particulate matter; however, in addition to the carcinogenic agents, urban-polluted air and cigarette smoke also contain irritant aerosols in the particulate phase. These adversely affect the respiratory epithelium and the overlying mucous blanket by producing a slowing, and occasionally total cessation, of flow. Combined analytical and physiological studies indicate that the irritant effect is a nonspecific one in that a variety of compounds produced the characteristic response. The action of irritants in facilitating localization and retention of particulates may serve the carcinogenic process by permitting abnormally long apposition of retained particles to the underlying epithelium and focal accumulation of greater quantities of carcinogenic agents. Because of the theoretical necessity that carcinogenic agents in an adsorbed state, as on soot, be in contact with target cells for a sufficiently long period to permit elution and entry into the cells, interference with the normal flow of the mucous stream can be postulated as a link in the pathogenetic sequence of bronchogenic carcinoma. Further, we have shown experimentally that the simultaneous or sequential inhalation of noncarcinogenic irritants and carcinogenic agents provides part of the necessary environment for tumor initiation in mice and rats (*12–14*). Subsequent to demonstrating the effect of irritant aerosols on ciliated mucus-secreting epithelium, we wished to determine whether this effect could be reversed or, more ideally, prevented by the use of pharmacologic agents. Kordik *et al.* (*15*) reported that ciliary action and production and secretion of mucus are controlled by acetylcholine. Because of potential significance from the viewpoint of preventive medicine, we investigated the possibility of attaining our goal by modification of this parasympathetic effect. A series of experiments was performed to determine whether the parasympathetic effect could be quantitated, and whether a technique for the use of parasympatheticomimetic agents could be developed in a manner consistent with clinical usefulness for neutralization of the effect of irritant aerosols on the respiratory epithelium.

## MATERIALS AND METHODS

The equipment and the laboratory procedures used for the exposure of epithelial segments and the measurement of particle transport have been previously described in detail. For reasons already published, the ciliated mucus-secreting epithelium of the esophageal tract of the frog was used in the experiments to be presented. Parallel studies with mammalian and amphibian epithelium of like histological morphology have demonstrated that the qualitative and quantitative findings from each are wholly consistent.

The characteristic response of mucous stream flow to irritants has already been reported, and the effects described in previous publications (*1*, *2*) serve as the basis for the current study.

*Pharmacologic agents.*—Three groups of compounds were used to obtain a parasympatheticomimetic effect: 1) compounds related to acetylcholine (acetylcholine bromide, methacholine chloride, bethanechol chloride, and carbachol), 2) cholinesterase inhibitors (eserine salicylate and prostigmine bromide), and 3) the alkaloid arecoline hydrobromide, and the related synthetic compound, 1,1-dimethyl-4-methoxycarbonyl-1, 2,3,6-tetrahydropyridinium iodide.

*Method of application.*—Exposure of the epithelium was accomplished by three techniques: 1) The agents were applied to the epithelium by dropper, and contact was permitted for 1 minute. Complete removal was effected by the epithelial surface being rinsed with Locke's solution. 2) The agents were also applied to the epithelium by dropper where they remained in contact with the epithelial surface throughout the experiments. The initial reading was taken 30 seconds after the drug was applied to determine the effect on baseline flow. Subsequent readings were taken as with all methods of exposure. 3) The agents were also applied to the epithelium as a spray with Freon as the vehicle (specially prepared by Riker Laboratories, Los Angeles, Calif.). Exposure of the epithelium was followed in 30 seconds by the first determination of the rate of flow.

*Dose.*—The concentrations of the agents used were determined on a molar basis. When applied as a rinse, a 1:10,000 dilution was used for all agents tested except acetylcholine bromide for which a 1:100,000 dilution was used. A concentration of 10 times this was used when the test agents were applied by the drop technique. When applied as a spray a concentration of 50 $\mu$g was delivered per application except for acetylcholine when the quantity was 6 $\mu$g.

*Measurement of rate of flow.*—The rate of flow of the mucous stream was measured by the transport of soot particles on the epithelial surface. Particles of carbon black were dropped from a small camel's-hair brush onto the mucous stream overlying the ciliated epithelium. The transport of the carbon particles was observed through a stereomicroscope fitted with oculars scaled with 10 divisions per mm at a magnification of 16×. Passage of the particles over the scaled area was timed by a stop watch,

and the rates were expressed in distance traveled per time unit, *i.e.*, mm per minute. The rates presented are the average of 10 individual readings. Control studies were performed in accordance with the technique already described (*1*). Although in earlier studies both air pollutants and cigarette smoke were used as the source of the irritant agents, this report is concerned exclusively with the use of cigarette smoke. The latter has the advantage of being subject to more exact technical control and therefore greater reproducibility of data. More critical, however, in the selection of cigarette smoke were the data from earlier studies which indicated that its effect on the rate of mucous flow decreased in intensity with successive applications. A suggested explanation was that a depletion or attenuation of some protective factor might have occurred. Since one of these known to be related to mucous secretion and ciliary activity is parasympathetic activity, we selected for investigation pharmacologic agents with parasympatheticomimetic properties to determine whether the adverse effect of cigarette smoke could be modified. Cholinergic agents or compounds that interfered with cholinesterase activity were used to 1) determine whether they exert a neutralizing or remedial effect on the adverse response of the epithelium, 2) determine whether they provided protection when applied prior to the application of cigarette smoke, 3) measure the duration of protection, 4) compare the effectiveness of the various modes of application, and 5) compare the effectiveness of the various agents.

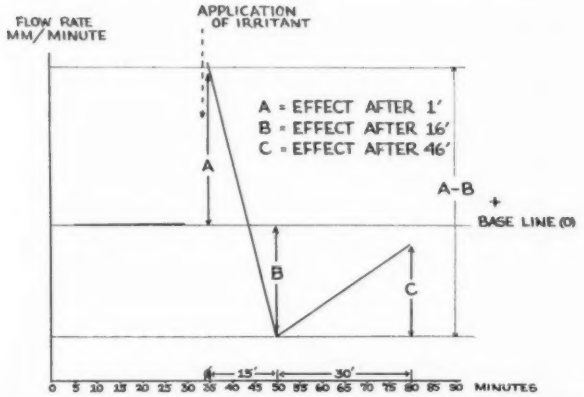
## RESULTS

### Response of Epithelium to Irritants

Prior to presentation of our data on the effect of parasympatheticomimetic agents, the previously described typical response of ciliated mucus-secreting epithelium to the application of an irritant is shown in text-figure 1(*1*). The initial accelerating response measured 1 minute after exposure is shown in text-figure 1 as A, and will be so referred to throughout the text. The inhibiting response occurring 16 minutes after initial exposure is shown in text-figure 1 as B and will be so referred to throughout the text. In all studies on the effect of irritants, the fastest, uniformly moving particles located in the axial stream were selected for the determination of the rate of flow of the mucous stream and, where possible, the ciliary activity.

### Effect of Concentration of Pharmacologic Agents on Mucous Flow

The toxicologic properties of the pharmacologic agents made it necessary to determine the range of concentration that would produce the desired effect on the epithelium and the relationship of the dose, preferably the optimal one, to the systemic toxicity of the agents. In text-figures 2 and 3 the average rate of increase in rate of flow after the application of




TEXT-FIGURE 1.—Characteristic response of mucous flow to test agents measured at 1, 16, and 46 minutes after exposure. A = effect after 1 minute; B = effect after 16 minutes; C = effect after 46 minutes; A−B = decrease of flow between peak of stimulation and maximum inhibition. *Note* uniform baseline flow rate between 5- and 30-minute intervals during which repeated readings were made.

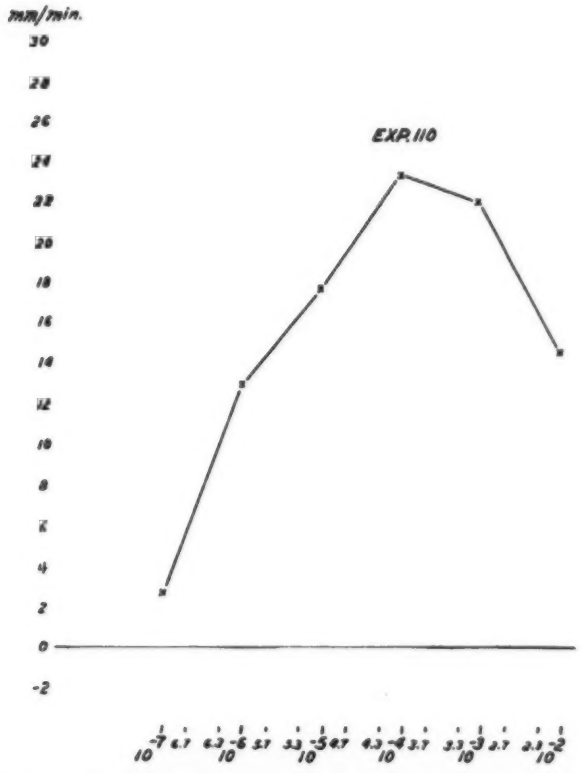
acetylcholine and eserine salicylate is given. A variation in response is related to the concentration of the agents, but, within the concentration ranges tested, the effect on the flow of the mucous stream was always an enhancing one, with the rate greater than the control level.

### Effect of Cholinergic Agents

A comparison of the effect of cigarette smoke on the rate of mucous flow of excised esophagus of the frog, pretreated with acetylcholine, the agent selected as representative of this group, is shown in text-figure 4. The characteristic accelerating effect on particle transport usually seen at A was affected but little when acetylcholine was used as the pretreatment agent. The inhibiting effect on the rate of mucous flow observed at B was obliterated. In essence, pretreatment effectively obviated the adverse effect of cigarette smoke. The data from an individual experiment are graphically presented in text-figure 5. As noted earlier, the typical response of the epithelium to an irritant is not entirely destroyed but rather is reduced in magnitude, and throughout the experiment the rate of flow is above that of the control or baseline rate of flow. While the experimental data presented are those in which acetycholine was used as the rinse, wholly comparable data result when all the cholinergic agents are used, independent of the mode of application.

### Effect of Cholinesterase Inhibitors

Eserine salicylate was chosen as a representative cholinesterase inhibitor. The results after its use are presented in text-figure 6. Little effect was discernible at A; however, the inhibiting effect of cigarette

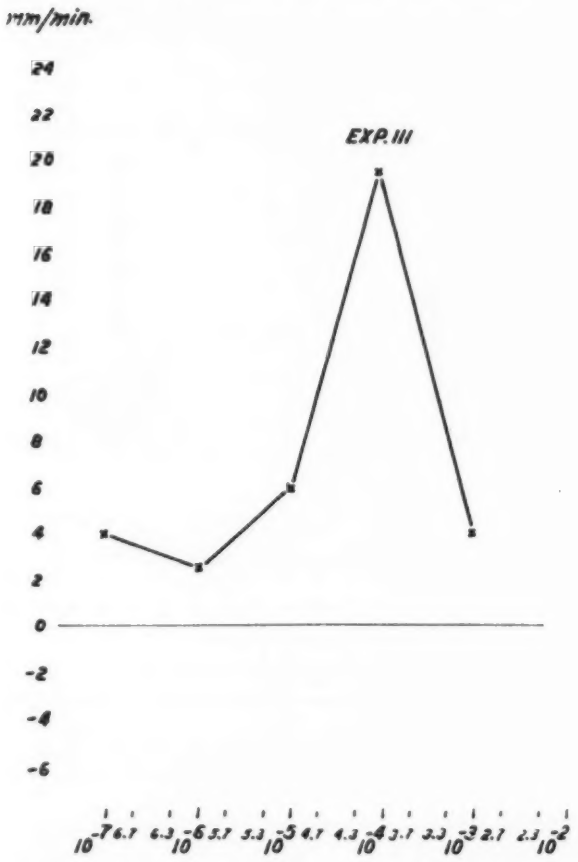



TEXT-FIGURE 2.—The effect of concentration of acetylcholine bromide on rate of flow of mucus on esophagus of the frog. Abscissa = molar concentration. Ordinate = rate of flow in mm per minute.

smoke seen at B in the control was obliterated by the pretreatment. In text-figure 7 data from a typical experiment are graphically presented with findings essentially identical to those discussed under cholinergic drugs. As with the latter, the effect was consistent, independent of the agent or mode of application.

### Effect of Arecoline and a Related Synthetic Compound

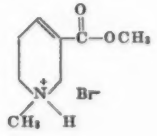
The compound arecoline is known to possess parasympatheticomimetic properties. We wished to determine whether an alkaloidal agent would produce an effect similar to that described for the cholinergic agents or cholinesterase inhibitors. As with both, arecoline effectively prevented the slowing of mucous flow when used in the pretreatment of the epithe-

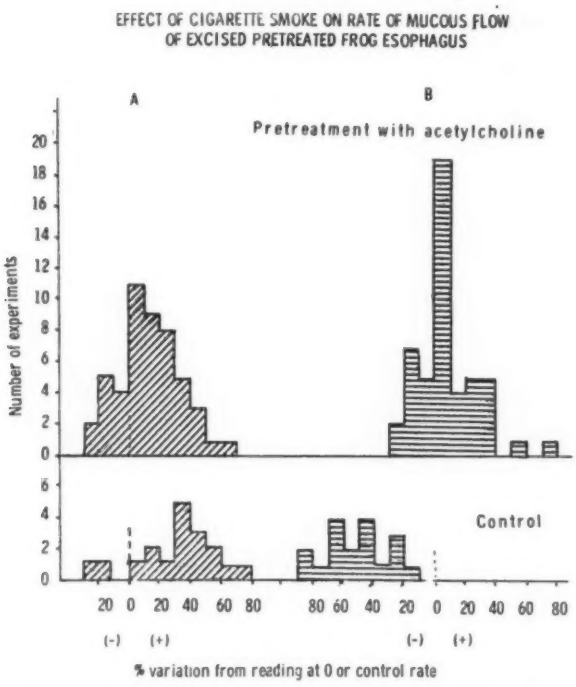



TEXT-FIGURE 3.—The effect of concentration of eserine salicylate on rate of flow of mucus on esophagus of the frog. Abscissa = molar concentration. Ordinate = rate of flow in mm per minute.

lium (text-fig. 8). A synthetic agent was also studied, and its effect was similar to that of all the other agents (text-fig. 9). Both compounds are related to nicotinic acid, and the formulas for each follow:

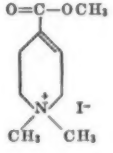
Arecoline = methyl 1,2,5,6-tetrahydro-1-methylnicotinate hydrobromide




EFFECT OF CIGARETTE SMOKE ON RATE OF MUCOUS FLOW
OF EXCISED PRETREATED FROG ESOPHAGUS
A
B
Pretreatment with acetylcholine
Number of experiments
Control
20 0 20 40 60 80
80 60 40 20 0 20 40 60 80
(-) (+)
(-) (+)
% variation from reading at 0 or control rate

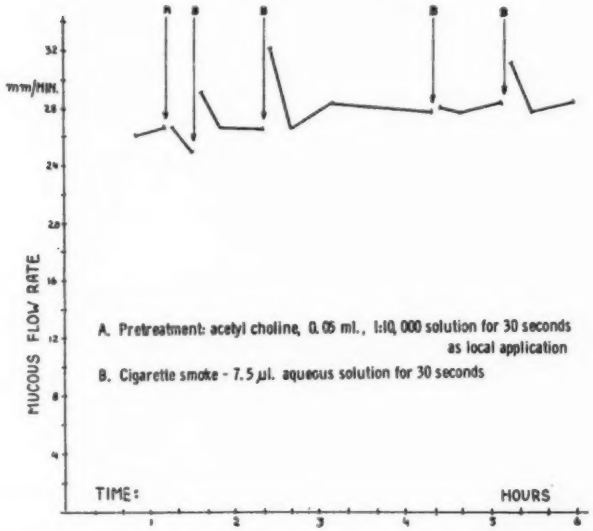
TEXT-FIGURE 4.—The effect of cigarette smoke on rate of mucous flow of excised, pretreated esophagus of the frog. Abscissa = percent variation from reading at 0 or control rate. Increase in the rate of flow is recorded to the *right* of the 0 reading, while decrease is noted to the *left*. Ordinate = number of experiments.

Synthetic agent = 1,1-dimethyl-4-methoxycarbonyl-1,2,3,6-tetrahydropyridinium iodide

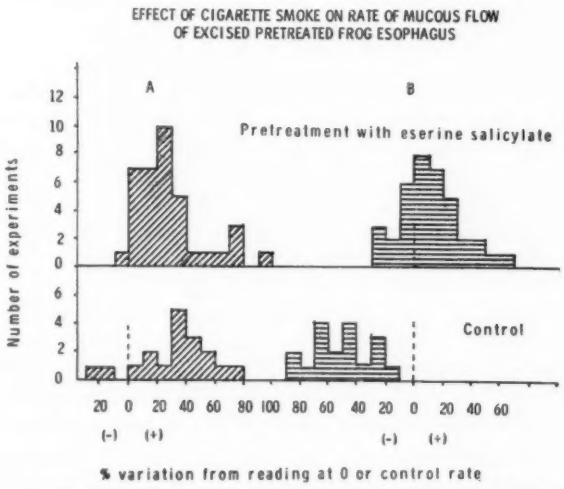



## DISCUSSION

Any appraisal of the practical implications of our findings requires consideration of the physiologic and pharmacologic bases of ciliary action, production of mucus, and rate of mucous flow. In an elegant study, Kordik and her associates demonstrated that the production of acetylcholine controls ciliary movement in the epithelium of the rabbit trachea. Our data confirm these findings and further establish that parasympatheticomimetic activity is crucially concerned with the rate

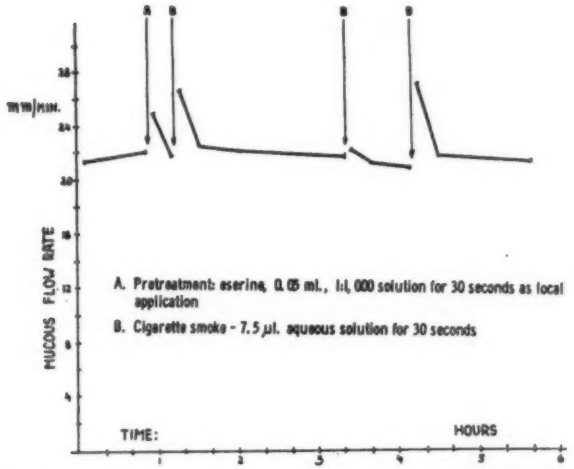

MUCOUS FLOW RATE
mm/MIN.
A. Pretreatment: acetyl choline, 0.05 ml., 1:10,000 solution for 30 seconds as local application
B. Cigarette smoke - 7.5 µl. aqueous solution for 30 seconds
TIME:
HOURS

TEXT-FIGURE 5.—Effect of cigarette smoke on rate of mucous flow of excised, pretreated esophagus of the frog.

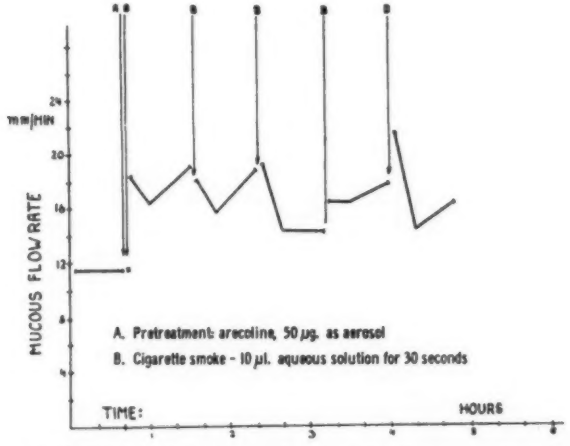

EFFECT OF CIGARETTE SMOKE ON RATE OF MUCOUS FLOW OF EXCISED PRETREATED FROG ESOPHAGUS
A
B
Pretreatment with eserine salicylate
Control
Number of experiments
% variation from reading at 0 or control rate

TEXT-FIGURE 6.—The effect of cigarette smoke on rate of mucous flow of excised, pretreated esophagus of the frog. Abscissa = percent variation from reading at 0 or control rate. Increase in the rate of flow is recorded to the *right* of the 0 reading while decrease is noted to the *left*. Ordinate = number of experiments.

of flow of the overlying mucous stream. Finally, both studies support the concept that the epithelial response is initiated and controlled by local cellular factors. This is evident from the following: Tracheal epithelium from the rabbit contains both choline acetylase and "true"

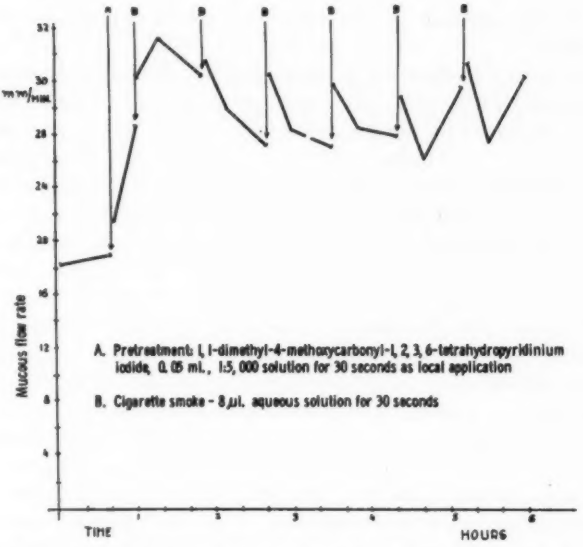



TEXT-FIGURE 7.—Effect of cigarette smoke on rate of mucous flow of excised, pretreated esophagus of the frog.




TEXT-FIGURE 8.—Effect of cigarette smoke on rate of mucous flow of excised pretreated esophagus of the frog.

cholinesterase, and acetylcholine in minute amounts is synthesized and liberated by these cells (*15*). Ganglion cells, a possible source of this agent, are not found in the tracheal mucous membrane. Results from studies with isolated epithelial segments and those with preparations with intact circulation and neural supply are comparable, and, despite limited quantitative differences in the response of the epithelium and the flow of mucus to the three classes of agents studied by us, the qualitative effect is a consistent one, *i.e.*, a protective effect. Our findings are


Mucous flow rate
TIME
HOURS
A. Pretreatment: 1, 1-dimethyl-4-methoxycarbonyl-1, 2, 3, 6-tetrahydropyridinium iodide, 0.05 ml., 1:5,000 solution for 30 seconds as local application
B. Cigarette smoke - 8 µl. aqueous solution for 30 seconds

TEXT-FIGURE 9.—Effect of cigarette smoke on rate of mucous flow of excised pretreated esophagus of the frog.

most readily attributable to the state of balance between acetylcholine stimulation and cholinesterase inhibition.

*Comparisons* in vivo *and* in vitro.—The effect *in vivo* of acetylcholine can be duplicated experimentally by the application of minute doses of acetylcholine to the mucous membrane. The action of cholinesterase as an antagonist can be observed after the removal of acetylcholine from the epithelium by washing. The resulting relatively local excess esterase is immediately manifested by a marked drop in rate of mucous flow. In addition to controlling ciliary movement, acetylcholine and esterase modify mucous stream flow by alterations in the viscosity of the mucus. Dilute, relatively nonviscid mucus of low specific gravity is secreted during the acetylcholine phase, while under the influence of the cholinesterase the mucus secreted is thick, tenacious, markedly viscid, high in specific gravity, and low in water. After transient esterase hyperconcentration, up to 45 minutes are required before acetylcholine-cholinesterase equilibrium is restored, and a return to normal viscosity and rate of mucous flow are noted.

A final corroboration of the specificity of parasympatheticomimetic effect was obtained when acetylcholine-like agents were used. It is of interest that these agents, which are poorly, if at all, hydrolyzed by cholinesterase, appear to bind the enzyme so that when the former are rinsed off the epithelium, the concomitant marked drop of the mucous flow rate effect observed earlier with acetylcholine did not occur. Presumably the inactivation of esterase by the acetylcholine-like homologues

permits the persistence of the effect of the continuously synthesized acetylcholine.

The duration of effect of parasympatheticomimetic agents persisted for periods up to 6 hours, and, while quantitative variations in response were occasionally noted, the protective effect was observed throughout the entire interval. The buffering effect was manifestly independent of the mode of application of the various agents, and there appeared to be little advantage in one compound over the other. Finally, our conclusions are congruous with those of Kordik in that low concentrations were relatively more effective in producing the effect than high concentrations; very high ones were totally ineffective.

At this time it is difficult to state with certainty whether the parasympatheticomimetic effect is mediated primarily through the effect on ciliary movement or on the concentration of mucus and its viscosity. While Kordik and associates lean strongly to the concept that the ciliary response is the primary avenue of action, our studies suggest that modification of mucous secretion and production is of equal significance in a determination of the response of the epithelium to irritants as well as to pharmacologic agents. We recognize that cilia are the motive force behind the flow of the mucous stream; however, we and others have observed situations in which actively beating cilia are inadequate to the task of propelling highly viscid mucus while less actively beating cilia can propel a watery mucous stream at physiologic or accelerated rates. Current intensive studies on the mechanisms of mucous secretion and the biochemistry of mucus are evidence of our concern with this factor.

In earlier studies we reported in detail the chemistry of soot recovered from polluted urban atmosphere (*11*). We also investigated the anatomic and metabolic fate of carcinogenic polycyclic aromatic hydrocarbons in mice and rats (*16*). These hydrocarbons occur adsorbed on soot particles in polluted air, and subsequent to their respiration and deposition on the respiratory epithelium a time interval is necessary for their elution of carcinogenic hydrocarbons from the particles (*17*). The liberated compounds are capable of entry into the epithelial cells of the tracheobronchial tree. The action of irritants in facilitating prolonged retention has been discussed. In addition, we previously demonstrated that the irritant aerosols responsible for alterations in mucous flow also cause desquamation of the superficial epithelium at a time when goblet-cell hyperplasia is so extensive and pooling of mucus so great as to be inconsistent with survival of the epithelium. The denudation of the superficial epithelium permits the immediate apposition of inhaled particulate carcinogenic matter to the surviving layer of basal cells. The inherent physiologic stimulus to epithelial regeneration is as a result subject to the modifying effect of the carcinogenic agents in the immediate area. This phenomenon may recur periodically and the repetitive cycle of desquamation and regeneration in the presence of these agents appears to give rise to hyperplastic, metaplastic, and ultimately neoplastic change (*7*).

Despite the demonstration of carcinogenic agents in polluted urban air

and cigarette smoke, analysis of epidemiological data reveals that by far the greater majority of those exposed to this carcinogenic milieu do not develop pulmonary cancer. To us an attractive and plausible explanation for this epidemiological pattern is that local and systemic host factors are significantly concerned with the entry and action of carcinogenic agents. We are actively investigating the systemic factors through studies of the metabolism of carcinogenic hydrocarbons in the intact host, particularly in relation to the hepatobiliary system. On the local level we feel that ciliary activity and mucous stream flow are the considerations of major significance. Thus, while the quantity of carcinogenic agents in the environment may be constant, variations exist in the quantities breathed, and actual cellular entry is dependent upon abnormal retention and prolonged cellular apposition. Great physiologic variation exists in relation to this property. Attenuation of the normal physiologic defenses of the respiratory epithelium to any degree results in the enhancement of the likelihood of a carcinogenic effect. Earlier reports of carcinogenic bioassay appear to confirm this conclusion (*13*).

The studies currently reported were directed toward the establishment of a method of augmenting respiratory-tract defenses to irritants either by means of prophylaxis (buffering effect) or neutralization (therapeutic effect). A series of parasympatheticomimetic agents was selected for this purpose, and on the experimental level, as described, they exert the desired effect. The systemic toxicity of these agents and their pharmacologic and physiologic doses are well known. In the species studied, both in the findings reported earlier and in the data presented here, the agents were used in concentrations well below those responsible for systemic toxicity. The doses used were similarly well below the toxic dose for man. The potent clinical usefulness of these compounds as buffering agents will be dependent on the results of detailed qualitative and quantitative studies *in vivo*.

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# Radiation Response of Mammalian Cells Grown in Culture. II. Survival and Recovery Characteristics of Several Subcultures of HeLa S3 Cells After X Irradiation [1]

R. Z. LOCKART, JR.,[2] M. M. ELKIND, *and* W. B. MOSES, *National Cancer Institute,*[3] *Bethesda, Maryland*

SUMMARY

**Three subcultures of HeLa S3 cells, which are of human origin, were studied to determine their response to X rays. The ability of the cells to retain unlimited proliferation was determined by standard tissue-culture techniques for handling single cells. Cells were irradiated with 55 kv X rays delivered at 722 rad per minute. The three subcultures showed characteristic differences in their survival parameters. Two of the subcultures had average mean lethal doses (MLD) near 100 rad, while the third had an average MLD of 131 rad. A range of average extrapolation numbers from 1.4 to 4.9 was observed, and each subculture had a different but characteristic average value. The three subcultures also contained different chromosome complements. It was not possible to correlate either the MLD or the extrapolation number with chromosome complements. In spite of this, surviving cells of all three subcultures were capable of repairing sublethal damage starting immediately after X irradiation. Repeated recovery cycles were also demonstrable when sufficient lengths of time were allowed between doses. In addition to recovery, increases in the MLD of recovered cells in some instances caused the net survival obtained after a given total dose of X rays to be considerably greater when the same total dose was fractionated.—J. Nat. Cancer Inst. 27: 1393–1404, 1961.**

SEVERAL STUDIES (*1–5*) have been completed by the use of the single-cell techniques of Puck and his associates (*6*) to investigate the lethal effects of X rays. The two parameters generally determined from survival curves are the mean lethal dose (MLD), the dose which reduces survival by a factor of $\frac{1}{e}$ ($\simeq$0.37) in the exponential region of survival, and the extrapolation number ($n$) (*7*). The great sensitivity of mammalian cells, the observation that chromosome aberrations result from doses of X rays in the order of a single MLD, and other observations, led Puck *et al.* (*1–3*) to state that the chromosomes are the primary sites of lethal irradiation. They have presented still further evidence for this view by reporting a correlation between the chromosome com-

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plement of various cell lines and their extrapolation numbers. They (*1–3*) have reported that euploid human cells yielded survival curves with an extrapolation number of about 1.4, while the curves from aneuploid HeLa S3 cells gave numbers around 2.

Elkind and Sutton (*4*, *5*) reported that Chinese hamster cells also yielded "multihit" type survival curves, but reported a range of extrapolation numbers. They further showed that damage as a result of an initial X-ray dose could be repaired rapidly so that, after the onset of division, the progeny of survivors as single cells responded as if they had received no previous irradiation (*8*). Bases (*9*) showed survival curves for HeLa S3 cells in which one strain gave an extrapolation number of 2, while a second strain, on the basis of a few experiments, yielded an $n$ of from 4 to about 12.

The present work was undertaken to study more carefully the variations in X-ray response of HeLa S3 cells. In addition, we wanted to see if recovery occurred in these cells which are of human origin and to what extent the variations in extrapolation numbers would influence the amount of recovery.

## MATERIALS AND METHODS

Several separate subcultures of HeLa S3 cells were investigated. They were designated S3-1, S3-2, and S3-3 for the purposes of identification. They were obtained separately at intervals of about 8 months from the same parental S3 line maintained in continuous culture in the laboratory of Dr. Harry Eagle.[4] They were maintained in Eagle's basal medium (*10*) supplemented with 10 percent undialyzed human serum. No attempt was made to reclone any of the cells, and variations in clonal morphology were evident within each subculture.

The procedure for obtaining cell suspensions, attaching the cells to petri dishes, and irradiating them has been described in detail by Elkind and Sutton (*5*).

In general, cells grown as monolayers not yet confluent were put into suspension with 0.03 percent trypsin (Nutritional Biochemicals Corp., Cleveland, Ohio, 1:300). After counting in a hemocytometer, we added an appropriate number of cells to 10 cm petri dishes containing 12 ml of regular growth medium and allowed the cells to attach to the dishes by incubating them for 4 hours at 38° C in a humidified atmosphere of about 2 percent $CO_2$ in air. The medium was removed just prior to irradiation, and the irradiated cells and unirradiated controls were refed with from 12 to 35 ml of medium, depending on the cell concentration. The X-ray source, operated at 55 kv and 12 ma, was a tube with a 1 mm Be end window (OEG-60 Machlett). The X-ray beam was filtered by 0.175 mm Al. The estimated dose rate was 722 rad per minute (*5*).

[4] Strains S3-1 and S3-2 were kindly provided by Dr. Robert Bases, who had obtained them previously, and strain S3-3 by Dr. Harry Eagle.

Early in the work, we encountered difficulty in obtaining clearly defined surviving clones when the surviving fraction was less than 0.01. We overcame this by feeding the cells after irradiation with medium consisting of equal parts of regular growth medium and *HU*-15 medium as previously described (*4*, *5*). Surviving clones were scored after 12 to 14 days incubation at 38° C. At the higher doses, there was evidence of abortive colonies. All colonies which clearly resembled clones when viewed by eye, and which contained at least about 100 cells, were scored as survivors. Three or more petri dishes were counted and averaged for each dose. Agreement between replicate dishes was usually quite good, and standard errors were small.

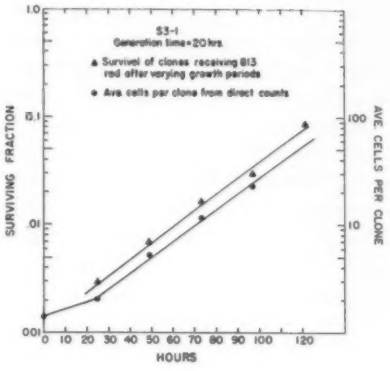
## RESULTS

### Sensitivity Independence

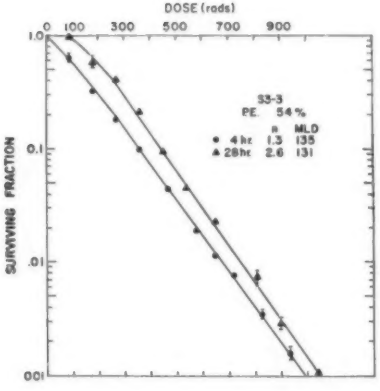
Two descriptive parameters of a survival curve are the MLD and $n$. As previously pointed out (*5*), if cells in groups survive independently, only $n$ should be affected by clumping which is frequently present to a small degree in tissue cells dispersed with trypsin. It was therefore necessary to ascertain if each cell reacts to X rays as an individual or whether its survival is influenced by its neighbors. Data showing that the surviving fraction is directly proportional to the number of cells per colony have been presented for Chinese hamster cells (*5*, *8*). Two kinds of experiments showing that this is probably also true for HeLa cells will be described.

In the first experiment, HeLa cells were plated on petri dishes. Then, at daily intervals, one set of plates was given a predetermined dose of X rays and the resulting number of surviving clones determined. A duplicate set, which received no irradiation, was fixed and stained, and the average number of cells per clone determined. We corrected the latter values by omitting clones with one and two cells after the 3d day of growth. The correction usually involved less than 10 percent of the clones counted and appears justified in view of the observed plating efficiencies. The effect of the correction on the doubling times was small. Text-figure 1 shows growth curves constructed from both kinds of data and illustrates that the probability that a clone would survive a given dose of irradiation increased at the same rate as the cells divided, which strongly suggests that each cell in a group maintained the same survival probability independent of growth.

A second type of experiment is illustrated in text-figure 2. HeLa S3-3 cells were plated and complete survival curves were determined after incubation for 4 hours and 28 hours at 38°. It can be seen that there is practically no change in the sensitivity of the cells after 28 hours' incubation, but that the survival curve is displaced upward by approximately a factor of 2. This suggests that by 28 hours cells had undergone one division and survived independently. The data are consistent with the


S3-1
Generation time=20 hrs
▲ Survival of clones receiving 813 rad after varying growth periods
● Ave. cells per clone from direct counts
SURVIVING FRACTION
AVE. CELLS PER CLONE
HOURS

TEXT-FIGURE 1.—Average number of cells per clone after the indicated time intervals at 38° C as determined by direct counting (*closed circles*), and the surviving fraction of clones after irradiation with 813 rad after the indicated time intervals at 38° C (*closed triangles*).

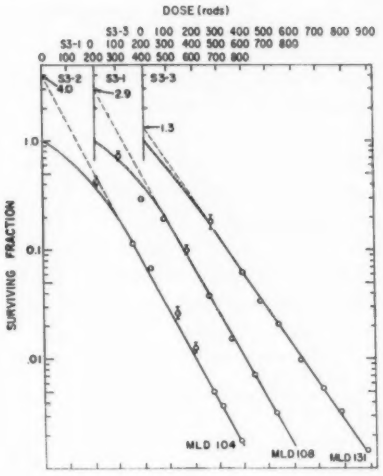



TEXT-FIGURE 2.—Initial survival curve for S3-3 cells after 4 hours at 38° C for attachment (*closed circles*), and the survival curve after 28 hours at 38° C (*closed triangles*).

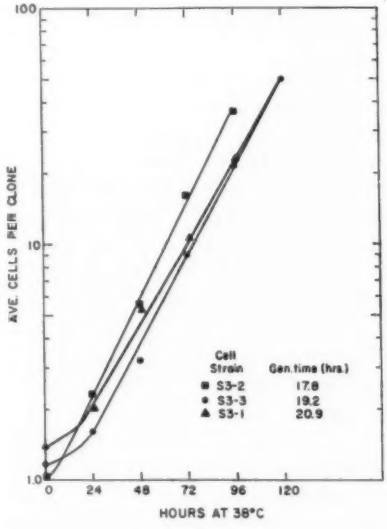
expected multiplicity at 28 hours obtained from typical growth curves of S3-3 cells (*see* text-fig. 4). The upward displacement of the survival curve shown in text-figure 2 plus the exponential increase in survival at a fixed dose shown in text-figure 1 strongly suggest that, for at least a 30-fold increase in average cell number per clone, the survival probability per cell is constant and independent of its neighbors.

### Survival Data of Several HeLa S3 Subcultures

Text-figure 3 shows representative survival curves obtained with the three subcultures of strain HeLa S3 cells. (Standard errors in the determination of survival are indicated in this and the other figures where they exceed the plotted points.) It is apparent that the three subcultures of cells showed differences both with regard to their sensitivities and extrapolation numbers. Table 1 shows the values obtained with the three lines in a number of experiments performed over a 6-month period. Small variations were noted in the values obtained in different experiments. All three subcultures gave essentially the same average plating efficiency of about 65 percent. While the sensitivity of S3-1 and S3-2 did




TEXT-FIGURE 3.—Individual, representative survival curves of HeLa S3 cells, subcultures S3-1, S3-2, and S3-3.




TEXT-FIGURE 4.—Growth curves of HeLa S3 cells, subcultures S3-1 (*closed triangles*), S3-2 (*closed squares*), and S3-3 (*closed circles*), constructed by a determination of the average number of cells per clone after the indicated time intervals at 38° C.

not differ significantly, S3-3 was somewhat more resistant. All three showed different average extrapolation numbers. These are presented in table 1, both as obtained from the plotted survival curves (column $n$) and after a correction for the initial multiplicity of plated cells (column $n/m$). The correction applies rigorously only if the multiplicity of the viable population is the same as that of the whole population, and cells in groups survive independently. These corrections were small for the S3-2 and S3-3 lines which grew loosely associated and which were well dispersed after trypsinization in contrast to strain S3-1.

TABLE 1.—Data from survival curves of HeLa cells

| Cell strain | Expt. No. | P.E.* (%) | MLD† | $n$‡ | $m$§ | $\frac{n}{m}$‖ |
|---|---|---|---|---|---|---|
| S3-1 | 19 | 43 | 108 | 3.1 | 1.41 | 2.3 |
| | 22 | 59 | 108 | 2.9 | 1.25 | 2.3 |
| | 23 | 60 | 100 | 5.0 | 1.26 | 3.9 |
| | 25 | 81 | 104 | 4.5 | 1.48 | 3.0 |
| Average | | 68 | 105 | 3.9 | 1.35 | 2.9 |
| S3-2 | 1 | 75 | 81 | 5.4 | 1.17 | 4.6 |
| | 4 | 70 | 104 | 4.4 | 1.09 | 4.0 |
| | 17 | 49 | 104 | 4.6 | 1.04 | 4.4 |
| | 18 | 60 | 90 | 7.6 | 1.04 | 7.4 |
| | 24 | 69 | 104 | 4.0 | 1.04 | 3.9 |
| Average | | 65 | 97 | 5.2 | 1.07 | 4.9 |
| S3-3 | 27 | 74 | 122 | 2.2 | 1.38 | 1.6 |
| | 28 | 60 | 126 | 1.4 | 1.17 | 1.2 |
| | 29 | 62 | 131 | 1.3 | 1.01 | 1.3 |
| | 31 | 54 | 131 | 1.5 | 1.02 | 1.5 |
| Average | | 63 | 127 | 1.6 | 1.14 | 1.4 |

*Plating efficiency.
†Mean lethal dose (rad).
‡$n$ = Extrapolation number.
§$m$ = Average number of cells per clone-forming unit.
‖Column $n/m$ indicates the extrapolation number corrected for cell clumping (*see* text).

In addition to the demonstrated differences in their response to irradiation, the three subcultures showed differences in their appearance in culture which permitted each to be identified by inspection. S3-1 exhibited large groups of cells that appeared somewhat smaller and very closely packed and which were difficult to disperse adequately by the standard trypsinization treatment adopted. S3-3 contained many cells that appeared smaller than those in strain S3-2 and which were also somewhat spindle-shaped with a tendency to form very spread-out aggregations of cells. Thus, S3-2 was conspicuous by its uniformity of large, loosely packed cells which were easily dispersed with trypsin.

An examination of the growth rate of these cell lines under single cell conditions showed no large differences. This is illustrated in text-figure 4. We constructed growth curves by determining the average number of cells per clone by direct counts at various periods after plating and correcting the values for plating efficiency as previously described.

Since the extrapolation number has been previously associated with the chromosome complement of other human cells (*2*), a study was undertaken to determine the distribution of chromosome numbers in the several cultures of HeLa cells under investigation.[5] The cytological methods used are described in (*11*). From the data in table 2, it can be seen that S3-2 cells showed a typically narrow distribution of chromosome numbers with

[5] The authors are indebted to Dr. Charles Greenblatt for preparing the cells for chromosome studies and to Dr. J. H. Tjio for determining the chromosome data shown in table 2.

a majority containing 78 (*12*, *13*). HeLa S3-1 cells had a narrow distribution with a modal number of 70 chromosomes. HeLa S3-3 cells probably had 69 to 71 chromosomes as its modal number, but it also differed from the S3-1 in that it had quite a wide range of chromosome numbers. HeLa S3-2 cells which contained the highest chromosome number had the highest average extrapolation number, 4.9. However, the other two cell lines that did not appear to possess significantly different modal values from each other nevertheless possessed different average extrapolation numbers, 1.4 and 2.9 and average MLD's, 105 and 127 rad. It is clear that no obvious correlation exists between the number of chromosomes contained in the three cultures of HeLa cells examined and the extrapolation numbers or MLD's obtained from their X-ray survival curves.

TABLE 2.—Distribution of chromosome numbers in HeLa S3 cells, subcultures S3-1, S3-2, and S3-3

| Cell strain | Chromosome number | | | | | | | | | | | | | Total cells |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 67 | 68 | 69 | 70 | 71 | 72 | 73 | 74 | 75 | 76 | 77 | 78 | 79 | |
| S3-3 | 10 | 13 | 25 | 16 | 18 | 15 | 3 | 3 | | | | | | 103 |
| S3-1 | | | 6 | 64 | 26 | 5 | | | | | | | | 101 |
| S3-2 | | | | | | | | 1 | 1 | 2 | 9 | 51 | 1 | 65 |

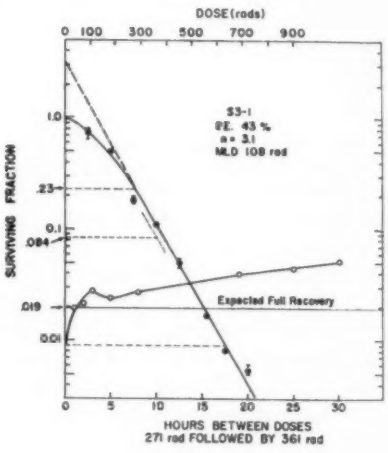
## Repair of Sublethal Damage in Surviving Cells

From the threshold-type survival curve, characteristic of mammalian cells irradiated and grown in culture, it can be inferred that cellular damage must be accumulated before unlimited proliferation is suppressed. This, in turn, implies that surviving cells are damaged cells with the amount of damage approaching a constant maximum as the exponential portion of the survival curve is approached. Elkind and Sutton (*4*, *5*), working with two cell lines of Chinese hamster cells, traced the radiation response of surviving cells starting immediately after exposure. They found that: 1) X-ray survivors rapidly repair their sublethal damage; 2) the primary evidences of damage and repair were the disappearance and reappearance of the threshold region of the survival curve; 3) repair processes start promptly and without any apparent delay; 4) the ability to repair sublethal damage was independent of the physiological state of the cells; and 5) the system(s) responsible for recovery is not attenuated by repeated exposures.

We have performed some typical recovery experiments to show that in a general way the three sublines of HeLa S3 cells described herein display recovery properties similar to those found in Chinese hamster cells (*4*, *5*, *8*). The main purpose of the experiments to be described is to show that fractionated exposures result in survivals which reflect primarily recovery and not postirradiation division. In view of the involved nature of some aspects of the postirradiation survival kinetics of Chinese hamster cells (*8*), a more complete comparison between the recovery properties of

HeLa cells and Chinese hamster cells will have to await a more detailed study.

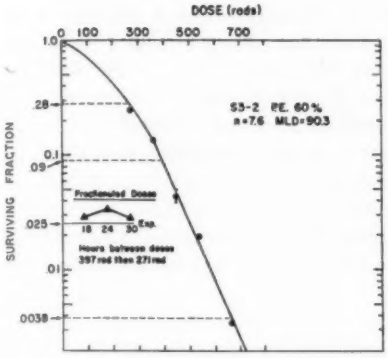
In text-figure 5, the *closed circles* trace the survival curve of lag-phase S3-1 cells irradiated after a 4-hour attachment period. The *open circles* trace the net survival resulting from the two doses, 271 and 361 rad, as a function of time at 38° C between doses. If both fractions were delivered at the same time (zero hrs between exposures), the net survival would have been 0.0090. Alternatively, if between exposures the survival curve of cells surviving the first exposure had returned to that of the initial population, the net survival to both exposures would have been $0.23 \times 0.084 = 0.0193$. The value of 0.084 is the corrected survival to the second exposure, corresponding to a survival curve for single cells rather than the plotted curve which is for cells having an initial multiplicity of 1.41 (*see 5*). This correction is approximate and therefore the survival level corresponding to full recovery is approximately 0.019. The fractionation curve shows that the net survival rapidly increased to this value within 2 to 3 hours, appeared to level off, and then continued to increase.




TEXT-FIGURE 5.—Initial survival curve for S3-1 cells after 4 hours at 38° C for attachment (*closed circles*), and the net survival to 271 rad followed by 361 rad as a function of time between exposures (*open circles*).

(In other experiments the minimum at about 5 hrs was more pronounced.) This latter increase may have been due to postirradiation growth since the rate of increase in survival is about equal to the growth rate of S3-1 cells (*see* text-figs. 1 and 4). Aside from the lag in growth which the control cells in this experiment probably experienced, because of at least the lag induced by the first dose, 271 rad, it is quite unlikely that any division had occurred by the time the net survival had reached the level corresponding to full recovery. The exponential increase in net survival from about 7 hours on is similar to observations described with Chinese hamster cells after relatively small first doses (*14*).

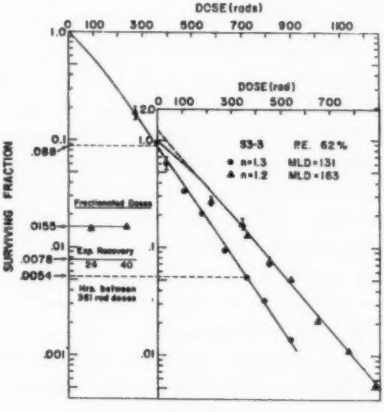
Text-figure 6 shows three points on a two-dose fractionation curve (*closed triangles*) for lag-phase S3-2 cells resulting from a first dose of 397




TEXT-FIGURE 6.—Initial survival curve for S3-2 cells after 4 hours at 38° C for attachment (*closed circles*), and the net survival to 397 rad followed by 271 rad at 18, 24, and 30 hours (*closed triangles*).

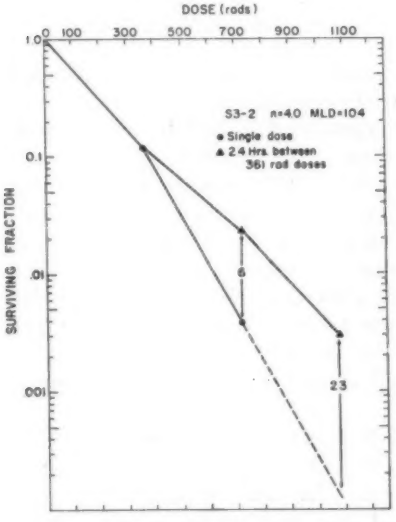
rad followed by second doses of 271 rad at 18, 24, and 30 hours after the first exposure. In this case, the net survival to both exposures at zero hours was 0.0038. The net survival corresponding to full recovery with no division after the first exposure is 0.025, if the survival curve of surviving cells was the same as the initial curve. Although the total division delay after the first exposure was not measured, it is possible that at least by 30 hours after 397 rad surviving cells had started to divide. We have found with Chinese hamster cells (*8*) that as the first exposure is increased, the net survival to a second exposure after the onset of postirradiation division may not increase in proportion to growth, as was suggested by the data in text-figure 5. Whether or not this situation obtained, it is clear that the net survivals observed are too large to be a reflection of postirradiation division alone.

Another illustration of the lack of dependence of the net survival to two doses on postirradiation growth is suggested by the data in text-figure 7. The *closed circles* trace the survival of lag-phase cells after a 4-hour attachment period. From the survival corresponding to 361 rad (0.088), the survival curve of surviving cells determined 24 hours later is plotted




TEXT-FIGURE 7.—Initial and fractionated survival curves for S3-3 cells: *closed circles*, initial survival curve after 4 hours at 38° C for attachment; *closed triangles*, survival curve of surviving cells 24 hours after a first exposure of 361 rad. The two *closed triangles* to the *left* of the survival curves represent net survivals to two 361 rad doses; the second doses were given at 24 and 40 hours.

(*closed triangles*). Although the MLD is larger, the latter curve has an extrapolation number essentially equal to the former. The two points plotted to the left of these curves were obtained with two 361 rad exposures; the second doses were given at 24 and 40 hours. Since the net survival expected was 0.0078 if the survival curve of recovered cells was the same as the initial survival curve, it is clear, in this instance, that the higher net survival at 24 hours resulted from the larger MLD for recovered cells at that time. That the net survival at 40 hours was the same as at 24 hours suggests that in this experiment the net survival did not reflect division, since by 40 hours it was quite likely that postirradiation division had been well under way.




TEXT-FIGURE 8.—Net survivals to successive 361 rad exposures for S3-2 cells. *Closed circles* represent survivals determined after 4 hours at 38° C for attachment. *Closed triangles* represent net survivals to two and three 361 rad exposures with 24 hours between successive exposures in each case.

As a final illustration of the effects of recovery on net survival, the results in text-figure 8 are shown. The *closed circles* are 2 points on the initial survival curve of S3-2 cells 4 hours after attachment. The first point is for a dose of 361 rad and the second for a dose of 722 rad. (Although the entire initial survival curve was determined to justify the extrapolation indicated by the dashed line, only the latter points are plotted since these are to be compared to the fractionation survivals.) The survival indicated by the first *closed triangle* resulted from two 361 rad exposures 24 hours apart, and the second *closed triangle* indicates the net survival to three 361 rad exposures 24 hours apart. Even if it is assumed that there had been no delays in growth resulting from the exposures or because the experiment was started with lag-phase cells, the ratios of the net survivals resulting from successive 361 rad exposures compared to single, equal total exposures would have been 2.6 and 6.5 at 24 and 48 hours, respectively (*see* text-fig. 4). Clearly, the ratios observed were too large to be due to postirradiation growth alone and must reflect

the influence of recovery between exposures. Alternatively, if after each 24-hour interval the survival curve was the same as the initial curve, the fractionation points would lie on a straight line through unity survival. The straight lines drawn between successive fractionation exposures would not depart significantly from a single straight line through the origin which fared all the data.

## DISCUSSION

Knowledge about the "sites" responsible for the loss of unlimited cellular proliferation resulting from X irradiation is being actively sought by many investigators. Up to the present, theories concerning the nature of these sites have been based mainly on survival and cytogenetic data (*1–3*).

This study points out that considerable caution must be observed if attempts are made to deduce X-ray-inactivation schemes from survival data for the following reasons. 1) Different subcultures of presumably the same cell line, namely, HeLa S3 cells, yielded survival curves whose average parameters differed from each other, and none of these subcultures yielded the same survival parameters as those first attributed to HeLa S3 cells (*1*). 2) A systematic inverse relationship between extrapolation number and MLD was observed with the three subcultures used. The reasons for this relationship are unknown. 3) All three subcultures yielded survival curves which appeared to represent homogeneous populations, *i.e.*, no inflection points or distinct changes in slope in the exponential regions, in spite of the fact that two of these subcultures, S3-1 and S3-3, had fairly broad distributions of chromosome numbers. 4) No obvious systematic relationship was apparent between chromosome number and survival parameters. 5) The properties of at least a cell line like HeLa S3, under conditions of continuous culture, are not invariant in time as displayed by changes in morphology in addition to changes in the other properties described.

Even though the survival properties of the three subcultures used were different, they all displayed the ability to recover between fractionated exposures. Whether the kinetics of recovery is qualitatively and quantitatively the same as that reported for Chinese hamster cells (*4, 5, 8, 14*) is not certain, though a number of similarities have been observable suggesting the kinetics may be the same. However, it is important to note: 1) Recovery has now been demonstrated in cultured cells from human and rodent origin and may therefore be a general property of mammalian cells; 2) the ability to display recovery does not require the rapid growth rates which Chinese hamster cells possess since HeLa S3 cells grow about half as fast (generation times; 9 to 11 vs. 18 to 21 hours, respectively); and 3) at least as concerns the qualitative aspects of recovery, the chromosomal content and/or number of a cell line do not appear to be relevant.

The lack of an apparent correlation between survival parameters and chromosomal constitution does not rule out the possibility that the sites of lethal action reside in the chromosomes since the relationship may be a subtle one. Nevertheless, in view of these results, it seems reasonable to suppose that the principal mechanism of X-ray-induced lethality may involve structures and systems other than the genetic apparatus alone.

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# Electron Microscopy of the H-1 Virus. I. Morphology of the Virus and a Possible Virus-Host Relationship [1, 2]

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SUMMARY

**An electron microscopic study of the H-1 virus, derived originally from a transplantable human tumor, shows that the virus consists of a dense core approximately 150 A in diameter. A coat 75 A wide, not made visible by the techniques used, may encircle the core, which makes the diameter of the "complete" virus 300 A. Virus particles have been observed enclosed in gray bodies in the cytoplasm of the Küpffer cells, in the interstitial cells of the kidney from 3- to 11-day-old infected hamsters, and in the chondrocytes of embryonic hamster cells incubated in media inoculated with the virus. Changes in the mitochondrial structure suggest that these organelles are intimately involved in a virus-cell interaction.—J. Nat. Cancer Inst. 27: 1405-1459, 1961.**

FRACTIONS AND cell-free filtrates of 8 transplantable human tumors, as well as livers, spleens, and other tissues derived directly from several cancer patients (*1*, *2*), produced a specific deformity in a large proportion of hamsters receiving, at birth, injections of any one of these materials. Neither mice nor rats were so affected. Serial passage in newborn hamsters of cell-free filtrates, prepared from the livers of 6-day-old infected animals, increased the virulence of the causative agent that was found to be a virus with osteolytic propensities (*3*, *4*). The teeth and bones of these hamsters were particularly affected (*5*). This report is a preliminary electron microscopic study of tissues from hamsters infected with the agent originally isolated from the transplantable human tumor, HEp-1. A strain of virus, isolated from the tissues of hamsters receiving injections of a deoxyribonucleic acid (DNA) preparation from frozen HEp-1 tumor, has also been studied in the liver of young hamsters and in embryonic hamster tissues incubated in media inoculated with the agent and, subsequently, grown in conditioned animals. There were particles approximately 150 A in diameter in the tissues of the infected hosts. Since

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similar particles were not observed in tissues from control animals, they are presumed to be the infective agent, designated H-1 virus.

## MATERIALS AND METHODS

*Source of virus.*—A 10 percent homogenate in distilled water of fresh transplantable human tumor, HEp-1 (*6*), was prepared in a Waring blendor and filtered through a 0.03 Selas filter impermeable to *Escherichia coli*. When this filtrate was injected subcutaneously into the nape of the neck of more than 300 hamsters within 6 hours after birth, 95 percent of the animals survived, and 98 percent of these were deformed. Serial passage in newborn hamsters of the livers from such 6-day-old infected hamsters (again prepared as a 10% homogenate in distilled water and filtered through 0.03 Selas filters) resulted in a rapid increase in titer so that the first-passage filtrate caused a number of early deaths, and injection of undiluted third-passage material resulted in death of the young hamsters within 4 to 5 days. Dilution of the third-passage filtrate to $10^{-8}$ produced no deaths, but 80 percent of the animals were abnormal. Livers for further passages from animals given injections of undiluted filtrate had to be removed by the 3d day of life because the infected hamsters seldom survived longer. First- and third-passage filtrates of the virus obtained in this manner directly from the tumor were used for 2 tissues described in this report.

A series of animals infected with DNA-derived material was also studied. DNA was prepared from frozen HEp-1 tumor [4] by the method of Kay, Simmons, and Dounce (*7*). This DNA preparation, inoculated into newborn hamsters, also produced an abnormality in almost 100 percent of the animals. Livers of such hamsters were prepared as a 10 percent homogenate in distilled water in the fashion described previously and passed serially into other groups of newborn hamsters. The titer rose rapidly as noted in the isolation of whole virus. First-, second-, and third-passage filtrates of DNA-derived material were used for 4 tissues examined.

*Tissues.*—Tissues from the anterior and posterior lobes of the liver and from the cortex of the kidney were excised from infected and control animals. Specimens of liver were removed from 2 or more hamsters killed at 8 or 11 days after their inoculation at birth with either first-passage filtrate containing the virus derived directly from the tumor ("direct" passage) or first-passage filtrate from animals receiving injections of DNA prepared from the tumor ("DNA" passage). Kidney tissue was taken from 3-day-old hamsters given injections of undiluted third passage of the "direct" line, and liver from 1- and 3-day-old hamsters given injections of undiluted third passage of the "DNA" material. In all cases, the infected animals were in a moribund condition when killed,

[4] By Dr. Ralph and Dr. Marion Barclay of the Sloan-Kettering Institute. Details of studies with DNA and ribonucleic acid (RNA) preparations from transplantable human tumors and the tissues of infected hamsters will be published by these authors and one of the present authors (H. W. T.).

except the 1-day-old hamsters. Tissues from 2 or 3 control animals in each group were examined. When some of the liver of the infected animals used for this study was tested in newborn hamsters for virus content, the inoculated animals died within 4 days. Filtrates of any of the control hamster livers caused no ill effects.

In another study, whole 13-day-old hamster embryo was finely minced with scalpels into medium 199 with 10 percent hamster amniotic fluid added and enough second-passage filtrate of the DNA-derived material to make a final $10^{-3}$ dilution of the filtrate. After a 15-minute incubation at 37° C, this material was injected into the cheek pouches of hamsters and the subcutaneous tissues of X-irradiated rats, all of which were given 3 mg of cortisone at the time of implantation. Eight days later, the small nodules present were excised and after several pieces had been removed for electron microscopic study, the remainder was finely minced and, without filtration, was injected into newborn hamsters. All babies died within 6 days. Control embryonic material without virus, similarly grown in conditioned animals, removed after 8 days and tested, did not produce any deleterious effects. The livers of the conditioned rat hosts carrying the virus-treated implants were also tested for virus in newborn hamsters and were found to be active. Livers of conditioned rat hosts, carrying untreated hamster embryonic material, were inactive.

*Fixation and embedding.*—The tissues were sliced into approximately 1 mm cubes in a pool of fixative and fixed for 1 hour at 4 to 5° C. The fixative used was 2 percent buffered osmium tetroxide containing sucrose (*8*). The tissues were then rinsed momentarily in distilled water and 10 percent neutral isotonic formalin and postfixed in the latter (*9*) for another hour. Dehydration in alcohol was carried out in the usual way at 4 to 5° C. The different alcohol dilutions sometimes contained 0.2 percent $MgCl_2 \cdot 6H_2O$ (*10*). Embedding was done either in 4 : 1 butyl and methyl methacrylate containing 0.5 percent azodi-isobutyronitrile as the catalyst (*11*), or in the mixture B of epon 812 described by Finck (*12*). In the latter, the final mixture was used at all stages of impregnation. The methacrylate was polymerized under ultraviolet light, and the resin was cured overnight at 40° C and for the following 24 hours at 60° C.

*Sectioning and electron microscopy.*—Tissues were sectioned with glass or diamond knives on either a Porter-Blum microtome or an LKB Ultrotome. Sections of the methacrylate-embedded tissues were mounted on collodion-coated grids that had been stabilized with carbon, whereas those of the epon-embedded tissues were mounted on uncoated grids. Lead hydroxide (*13*), lead subacetate, or lead acetate with a subsequent exposure to ammonia vapors (*14*) was used for staining sections that were examined in a Siemens Elmiskop I electron microscope, with a double-condenser system and a 50-$\mu$ aperture in the objective lens. Electron micrographs were taken at 10,000 to 40,000 instrument magnification and enlarged photographically as desired.

## OBSERVATIONS

Virus particles were observed in the cytoplasm of the Küpffer cells of the liver, the chondrocytes of cartilage, and the interstitial cells of the kidney from infected hamsters. The nuclei of these, as well as other cells in the liver and kidney examined in this study, did not reveal the presence of any virus particles.

### Küpffer Cells

The ultrastructural appearance of the Küpffer cells was essentially the same in the livers of normal and infected hamsters. The cells lining the surface of the sinusoid, either completely or partially, had the appearance of squamous cells. The plasma membrane, smooth and devoid of surface processes, was unlike the hepatic cells that had microvilli projecting into the perisinusoidal space (fig. 1). The nuclei were located at one end of the cells, with the chromatin material well-dispersed in the karyoplasm. Nucleoli were centrally placed. There were few mitochondria, randomly dispersed near the periphery of the cells (fig. 1). The endoplasmic reticulum, consisting of membranes with attached ribonucleoprotein (RNP) granules, had lamellar and vesicular profiles. Elements of this system, as well as free RNP granules, were disposed randomly throughout the cytoplasm. The Küpffer cells in normal as well as infected livers possessed an extensive Golgi complex (fig. 1), which exhibited all three components described by Dalton and Felix (*15*). Centrioles were observed occasionally in these cells.

In some Küpffer cells of the infected tissues, the ground substance of the cytoplasm exhibited low electron density. The endoplasmic reticulum appeared to have the vesicular profile only, and there were more vacuoles in the Golgi complex than are usually seen.

### Chondrocytes

The chondrocytes in embryonic hamster cartilage were oval (fig. 9). The karyoplasm of their nuclei, which were also oval, had a fine homogeneous granularity except at the periphery where there was an accumulation of granules. The nucleolus and the nuclear envelope were indistinct. In the cytoplasm, the sparse mitochondria had a structure similar to those observed in other mammalian cells (fig. 10). The Golgi complex was rarely seen, and the elements of the endoplasmic reticulum had elongated profiles. The most conspicuous feature of the cytoplasm of these cells was the great number of large lipide bodies (figs. 9, 10, and 11). Chondrocytes of the cartilage in the control hamsters differed from those in the infected tissue by the presence of an extensive endoplasmic reticulum and a large number of free RNP granules.

### Interstitial Cells in the Hamster Kidney

The interstitial cells in the hamster kidney had an attenuated appearance. The cell membrane was smooth and occasionally broken at a few points along its surface (figs. 14, 15, and 16). The nuclei were oval and sparsely filled with the chromatin material (figs. 14 and 15). The perinuclear space was frequently dilated. The outer nuclear membrane, which was studded with the RNP granules, sometimes extended into the cytoplasm to form elements of endoplasmic reticulum (fig. 14). In the cytoplasm, these latter organelles usually had circular profiles. Mitochondria, which were few in number, possessed the usual structure, but the ground substance showed a variable density (figs. 13, 14, 15, and 16). Round bodies, differing widely in their structures, were observed in the cytoplasm. While most of them were bounded either completely or partially by a single smooth membrane, a few of them showed segments of double membranes along their surfaces. The ground substance of some of these bodies was homogeneous and contained a few small vesicular bodies, while others had a granular matrix.

The scant cytoplasm, the dilation of the endoplasmic reticulum, and the loss of matrix in mitochondria in the interstitial cells of kidney were not a result of poor preservation of the tissue, since in the same tissue block the tubule cells did not exhibit any of these characteristics (fig. 25). The interstitial cells in normal kidney had a similar structure.

### Localization and Morphology of the Virus

In the cytoplasm of the Küpffer cells and the chondrocytes in the hamster-embryo transplant infected with the "DNA" passage, and in the interstitial cells in kidney from the 3-day-old hamsters infected with the "direct" passage, virus particles were observed within round bodies bounded by a single smooth membrane (figs. 1 through 15, 17, 21, 22, and 23). Comparable bodies have been called "gray bodies" (*16*) or "microbodies" (*17*) by some investigators. In a few chondrocytes in cartilage, the limiting membrane was either indistinct or ruptured, and free virus particles were seen in the cytoplasm (fig. 10). The gray bodies in the infected cells were usually less than 1 $\mu$ in diameter, *i.e.*, they lay within the range of variation of the circular profiles of mitochondria. Occasionally, in the Küpffer cells and the chondrocytes, these bodies assumed larger dimensions, sometimes comprising a major portion of the cytoplasm (figs. 7 and 11). The ground substance of the gray bodies, which was homogeneous, varied from very low to moderate in its electron density. Some of the gray bodies appeared to be completely filled with virus particles, while others had only a few in the plane of section. Occasionally, similar bodies without any virus particles in the plane of section were observed in the cytoplasm of the Küpffer cells and in the interstitial cells in kidney. In figure 6, three gray bodies, quite close to one another, are seen in the cytoplasm of a Küpffer cell. The gray body marked "1" in the figure appears completely filled, that marked "2" appears partially

filled with virus particles, whereas that marked "3" appears entirely devoid of any particles. In Küpffer cells, virus particles were localized occassionally along the periphery of a few bodies with little or no electron density (figs. 7 and 8). In the livers from 8- and 11-day-old infected hamsters, gray bodies were observed very rarely.

In the 4 samples of infected livers examined, no virus particle was ever seen in the parenchymal cells or free cells in the sinusoids. On the other hand, virus particles were observed in 2 instances in convoluted tubule cells of kidney from a 3-day-old infected hamster (fig. 25). The particles were localized, as in other cells described previously, inside a membrane-bound body and were distinct.

The virus particles appeared to consist of only a dense core, with a maximum diameter of 150 A, without any limiting or enclosing membrane.[5] Their regularity in shape distinguished them from the RNP granules that measured 180 to 220 A. The virus particles were spherical, while the RNP granules showed variations in their structure (figs. 3 and 6). In the gray bodies, which were completely filled with virus particles, the latter were quite distinct (figs. 3, 11, and 17). In figure 11, the virus particles, which occupy a large area of the membrane-bound body in the chondrocyte, are very sharp in some regions. In these regions each particle is surrounded by 6 other particles that appear to form a hexagonal pattern (fig. 12). These particles are always separated from one another by approximately 120 A. In gray bodies where the particles occupy a small area, there are few distinct particles (figs. 2 through 6).

### Variation in the Structure of Mitochondria

As described earlier, mitochondria in the Küpffer cells of the liver, the interstitial cells of the kidney, and the chondrocytes of the cartilage of infected hamsters were generally bounded by 2 membranes, the inner one of which was continuous with the cristae (fig. 1, M). Occasionally, in the Küpffer cells in the liver and the interstitial cells in the kidney, the profiles of these organelles showed an irregular organization of their inner membranous components (figs. 1 and 14, M1) or an absence of cristae (figs. 2 and 15, M2). A few mitochondria exhibited both of these characteristics. In the cytoplasm of these cells, bodies bounded by a single membrane and containing a few vesicles, which may have been derived from the inner mitochondrial membrane, were also observed (figs. 15, 16, and 17, M3). Scattered granules, approximately 150 A in diameter, were present in these bodies. It is therefore possible to visualize a gradual change in the mitochondrial structure according to the following transition: M→M1→M2→M3.

In the interstitial cells (fig. 18) and the tubule cells (figs. 19 and 20) of the kidney, and in the hepatic cells (fig. 24) of infected hamsters, mitochondria were occasionally observed enclosed by a double-membrane-

[5] It is unknown how much the dehydration of tissues at 4 to 5° C, epon embedding, and staining with lead compounds would change the size of the virus particles.

bound body. In figure 20, 2 or possibly more mitochondria are enclosed in a similar body. The ground substance of these bodies, which was variable in electron density, contained a few dense particles. In figure 20, these particles appear similar to RNP granules. In figure 18, however, their organization and appearance are typical neither of RNP nor virus particles, though they more closely resemble the latter. In a rarely observed instance, the cytoplasm of an interstitial cell of kidney contained 4 dense bodies bounded essentially by a single membrane (fig. 21). Two of these bodies, marked "1" and "2", enclose a mitochondrion whose matrix appears to fuse with that of the surrounding body at certain points as observed at a higher magnification (fig. 23) for one of these bodies. Dense particles, measuring about 150 A, fill most of such bodies (fig. 22).

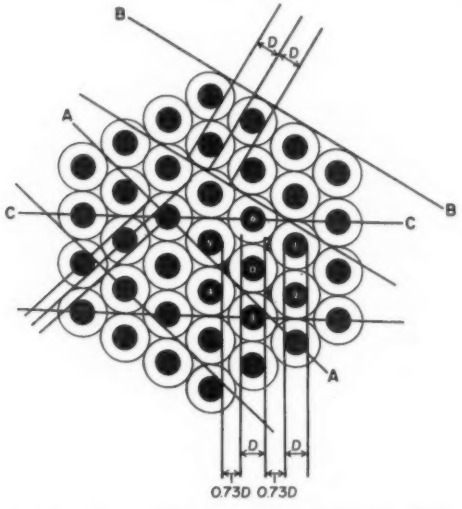
## DISCUSSION

### Morphology of the H-1 Virus

The virus particles observed in tissues from animals given injections of either the agent derived from the HEp-1 tumor or the DNA from the tumor are of identical morphology so far as this study shows. The structure of the H-1 virus suggests that it is a type-D virus as described by Bernhard (*18*). Other viruses in this category are the "K," polyoma, the Shope papilloma, the poliomyelitis, and the Coxsackie. The first 3 viruses have been observed in both nucleus and cytoplasm (*19–23*), and the last 2 in the cytoplasm of the infected cells (*24–26*). In an earlier study, intranuclear virus-like bodies in cells infected with poliomyelitis virus were also reported (*27*). Each of these viruses forms a crystalline array in the infected cells. The diameter of the individual virus particle in the poliomyelitis and Coxsackie viruses does not differ significantly from the center-to-center spacing of the adjacent particles in the crystalline array (*24*, *28*). In polyoma virus (*23*) and the adenoviruses (*29*), the diameter of an individual particle is less than the center-to-center distance. It has been suggested that a layer of material, not visible in osmium-fixed tissues, might exist around the dense particles seen in the electron microscope (*22*, *29*). A recent study on the polyoma virus shows that the diameter of a particle equals the center-to-center separation between the particles in a crystalline array (*30*). Whether this is true for all viruses arranged in crystalline arrays is unknown.

The H-1 virus, whether derived from the "direct" or the "DNA" passage, appears to be different, since no crystalline array of virus particles has been observed. The electron micrographs of this agent suggest that it is spherical. Even in those gray bodies where the virus can be assumed to be mature and where the particles occupy the entire volume of the body, they are separated from one another by a distance slightly greater than their radii (fig. 12). If the virus particles are spherical and occupy the entire volume of the body, they should appear adjacent to one another in

some areas of the micrographs. A lack of an observation of this kind suggests that the virus particles do not consist entirely of a dense nucleoid, but have an outer coat not visible in the electron microscope with the techniques used in this study. If we assume that this coat has a thickness equal to the radius of a virus particle, *i.e.*, the "complete" virus has a diameter of 300 A, and that they are closely packed in a certain volume, they will form a hexagonal pattern as shown in text-figure 1. If the plane of section corresponds to one designated AA, an electron beam will traverse through the same amount of nucleoid material at all points in the plane. No image of the particles will therefore be formed, and the corresponding area will appear "gray" in the electron micrographs (fig. 11). If the aggregates of virus particles are sectioned along the plane CC, there will be no "gray" area. The dark and clear areas will alternate with the result that the particles will appear distinct in the electron micrographs. The particles numbered 3, 0, and 6 will form one image that will be separated by the images corresponding to particles 4 and 5, and 2 and 1 by a distance $(\sqrt{3}-1)$ D or approximately 0.73 D, if D is the diameter of the dense core. This figure can easily be calculated from the geometry of the drawing. In each plane, above and below the plane of the paper, there are 3 more particles adjoining the one marked "0," above and below the points X, Y, and Z (text-fig. 1). The particles corresponding to points Y and Z will form one image and that corresponding to the point X, together with those adjoining 3 and 4, and 5 and 6, will form another image. In the electron micrographs, one particle will, therefore, appear surrounded by




TEXT-FIGURE 1.—Diagram illustrating spherical particles in a close crystalline packing. Each particle has a dense core of 150 A with a light coat 75 A in width about it. The size of the "complete" virus would therefore be 300 A. Thicknesses of the sections along the planes AA′, BB′, and CC′ vary between 500 to 600 A, which was the range of thickness for tissue sections examined in this study.

6 similar particles that will form a hexagonal pattern and be separated from one another by approximately 0.73 D or by 110 A as observed in figure 12. If the plane of section be parallel to that shown as BB in text-figure 1, particles of moderate electron density will appear adjacent to one another, as seen in the upper-right corner of figure 12 (single arrow). The 3 planes of section considered here produce either no image, a distinct image, or an image of moderate density. The other sets of planes will produce intermediate images.

The electron micrograph of H-1 virus, in particular those in figure 11 or where the particles are assumed to be mature, shows that the aggregation of the particles varies in different areas, *i.e.*, the characteristic pattern is not repeated. A crystalline aggregate, on the other hand, is formed by a repetition of a particular pattern. The observations on the H-1 virus suggest that particles are not formed around one "nucleus," if they are formed at all at these sites, but around a number of nuclei.

### Virus-Host Cell Relationship

The electron micrographs demonstrate two things: 1) a change in the mitochondrial structure and 2) a variation in the electron density of the virus particles within gray bodies in the cytoplasm of infected cells. No nuclear changes were seen in the particular cells observed in these studies. On the basis of the present observations, no conclusions are possible on the dynamics of the interaction between virus and cell. There is a suggestion that the virus "infects" the mitochondrion, which then loses its structure and forms a gray body that becomes the site of synthesis for new virus. On the other hand, the mitochondrial changes may be an end stage in a process in which the gray bodies have an early role. In any case, the electron micrographs indicate that mitochondria are most significant in the virus-host relationship.

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**Figures 1 to 12, and 24 show electron micrographs of cells in 2 tissues: liver from a 3-day-old hamster and cartilage from an 8-day-old transplant of hamster embryo, both infected with the "DNA" virus passage. Figures 13 to 23, and 25 show electron micrographs of cells in kidney from a 3-day-old hamster infected with the "direct" virus passage.**

PLATE 195

FIGURE 1.—Cytoplasm of Küpffer cell with mitochondria (M) randomly dispersed. Two mitochondria (M1) show irregular organization of the internal membrane. The Golgi apparatus (GA), consisting, for the most part, of lamellar and vesicular components, is extensive. Centrioles (C) are observed occasionally. The cell membrane (CM) of the Küpffer cells is smooth, while that of hepatic cells has microvilli (MV) extending into perisinusoidal space. Electron micrograph shows one gray body (GB) with its bounding membrane and two others tangentially cut. Virus particles (VP) are seen in these bodies. × 30,000

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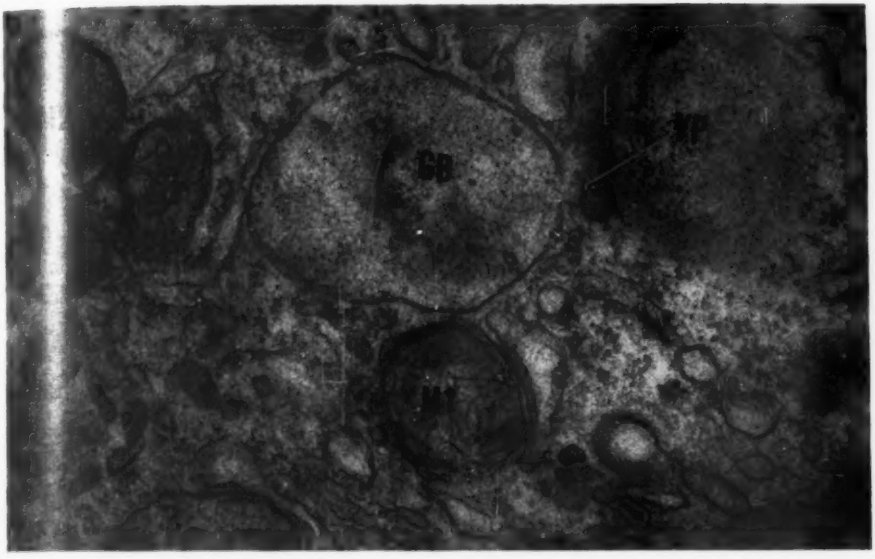
PLATE 196

FIGURE 2.—Small area of figure 1 at a higher magnification. Virus particles are seen distinctly. × 60,000

FIGURE 3.—Virus particles occupying only small area of gray body in plane of section shown. Endoplasmic reticulum (ER), with attached RNP granules, is seen in the cytoplasm. Virus particles are distinguishable from RNP granules when they are packed closely. The former are spherical while the latter show irregular morphology. × 100,000


GB

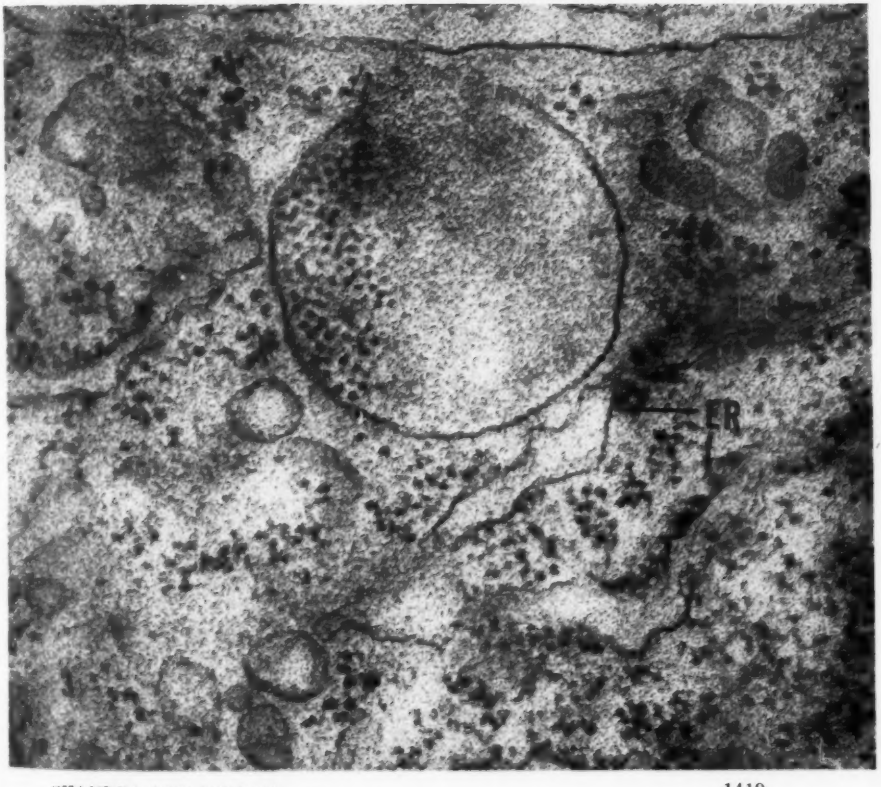
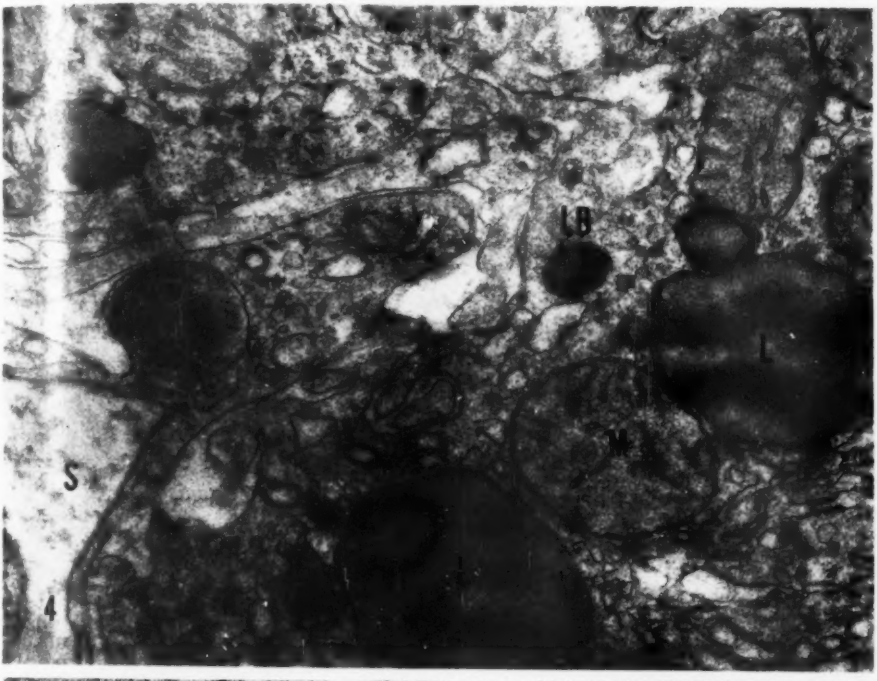

ER

PLATE 197

FIGURE 4.—Gray body (GB) containing virus particles in cytoplasm of Küpffer cell in liver sinusoid (S). Portions of 2 hepatic cells, having mitochondria (M), lipide bodies (L), and a lysosome body (LB) are seen in *upper right* and *lower left* corners. × 35,000

FIGURE 5.—Gray body in figure 4 at a higher magnification. × 160,000


LB
L
M
S
4

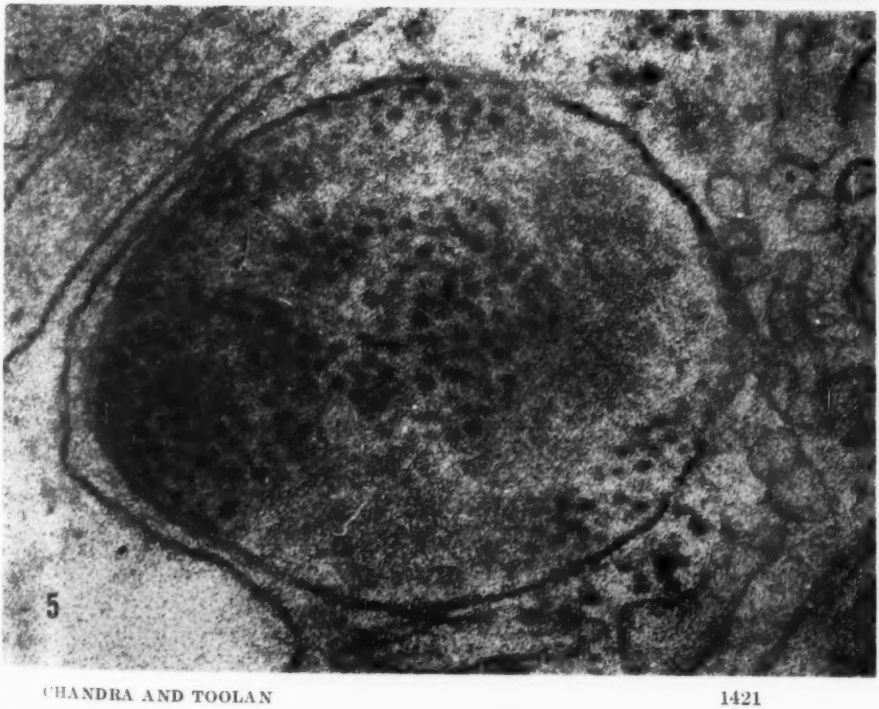

5

PLATE 198

FIGURE 6.—Three adjacent gray bodies in Küpffer cell. Gray body (GB1) appears completely filled, while other (GB2) is partially filled with virus particles. In plane of section, third body (GB3) is devoid of particles. Nucleus (N), with uniform distribution of chromatin material, appears in lower region. × 80,000

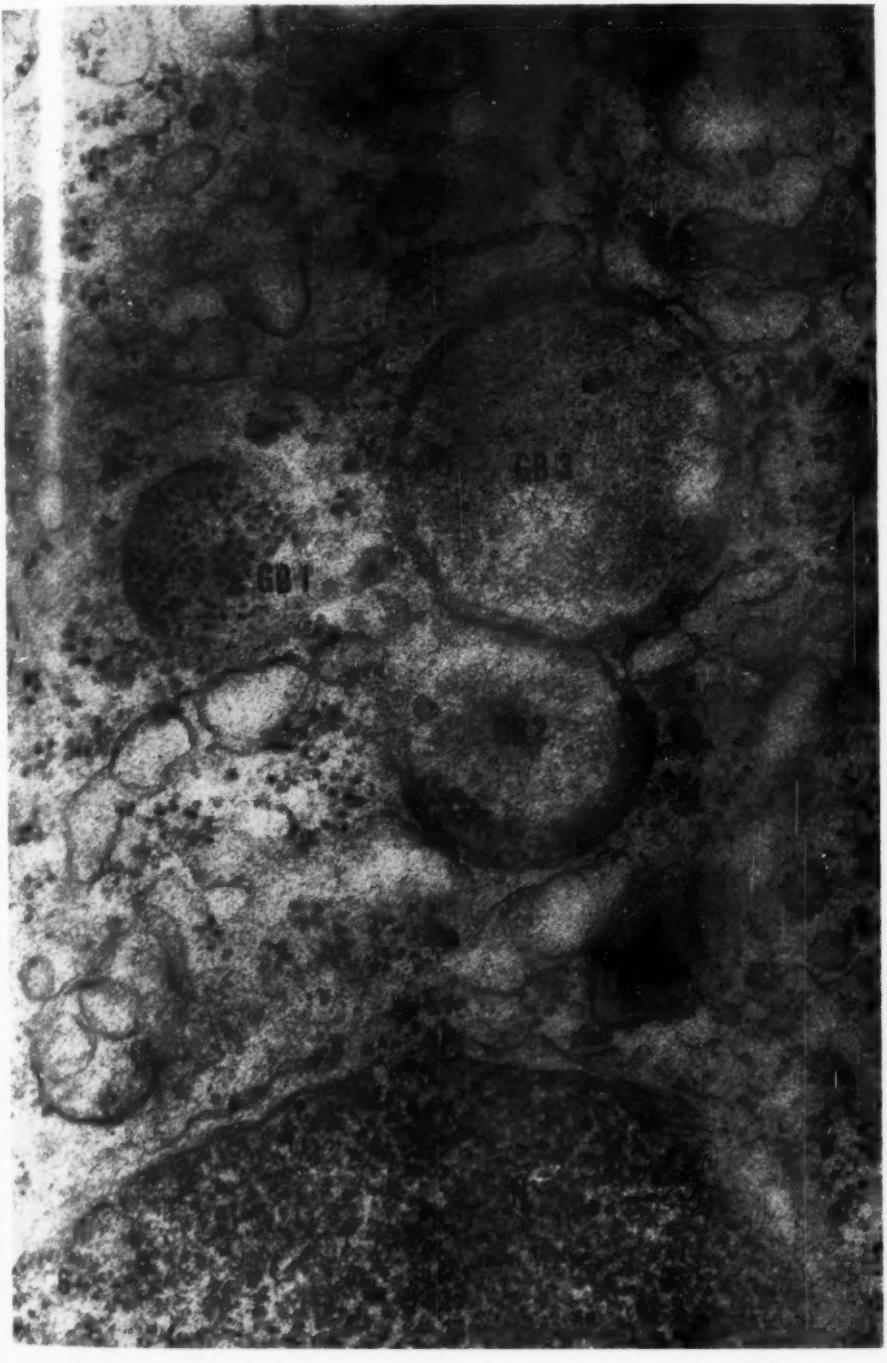
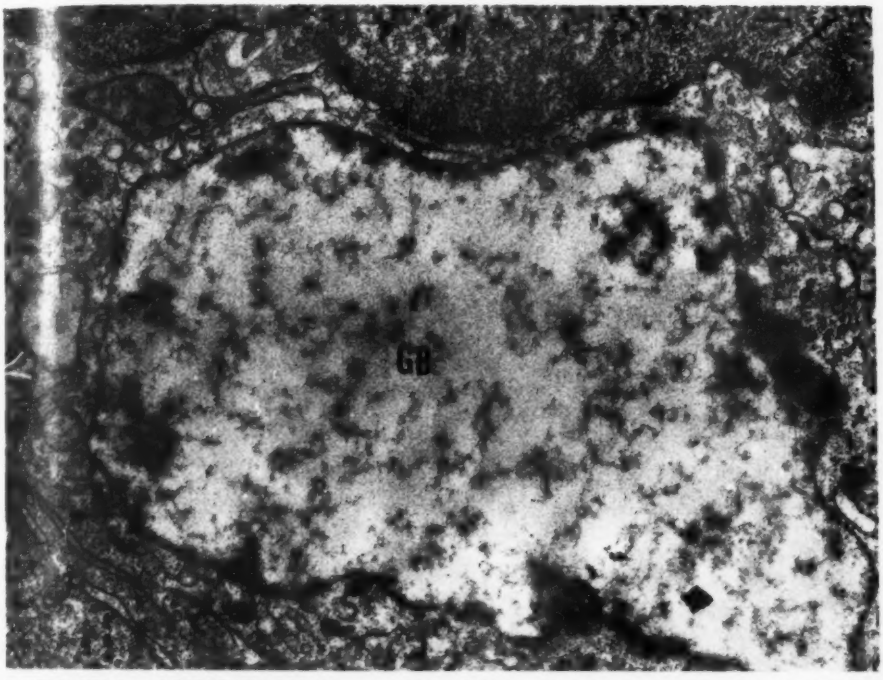

GB 3
GB 1

PLATE 199

FIGURE 7.—Large gray body (GB), which has appearance of a vacuole, contains virus particles lining inside of membrane enclosing the gray body. *Arrow* points to nuclear poles. × 24,000

FIGURE 8.—Segment of gray body in figure 7 at higher magnification. × 60,000


GB

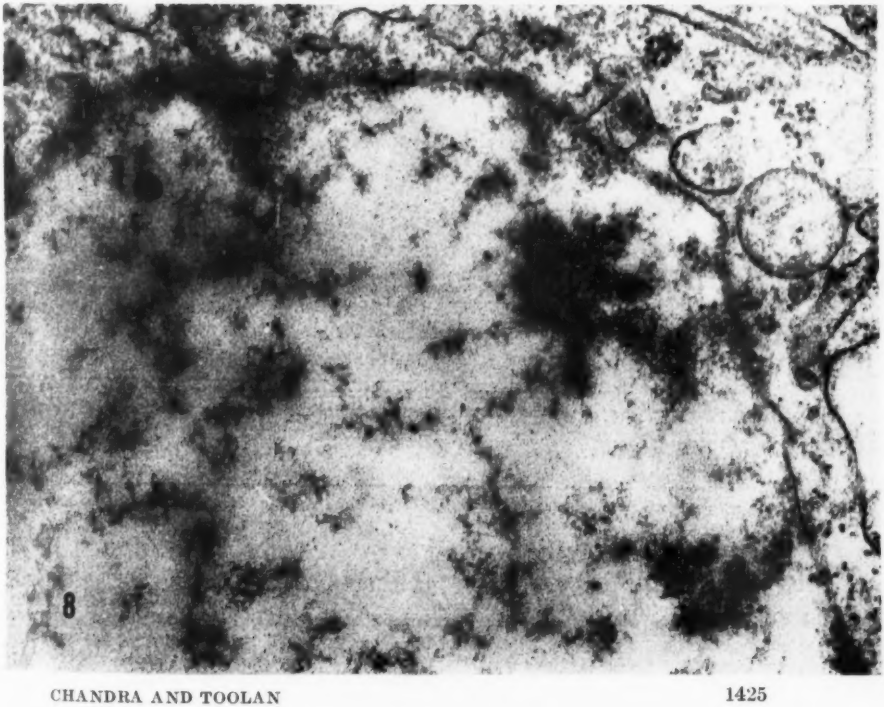

8

PLATE 200

FIGURE 9.—Portions of 2 chondrocytes are seen in the electron micrograph. Nucleus (N) is homogeneous with a little margination of chromatin material. Mitochondria, few in number, are not seen in this electron micrograph. Endoplasmic reticulum has lamellar profiles only. Lipide bodies (L) are abundant. Gray bodies (GB) are dispersed randomly. Collagen fibers (CF) fill matrix surrounding cells. × 32,000

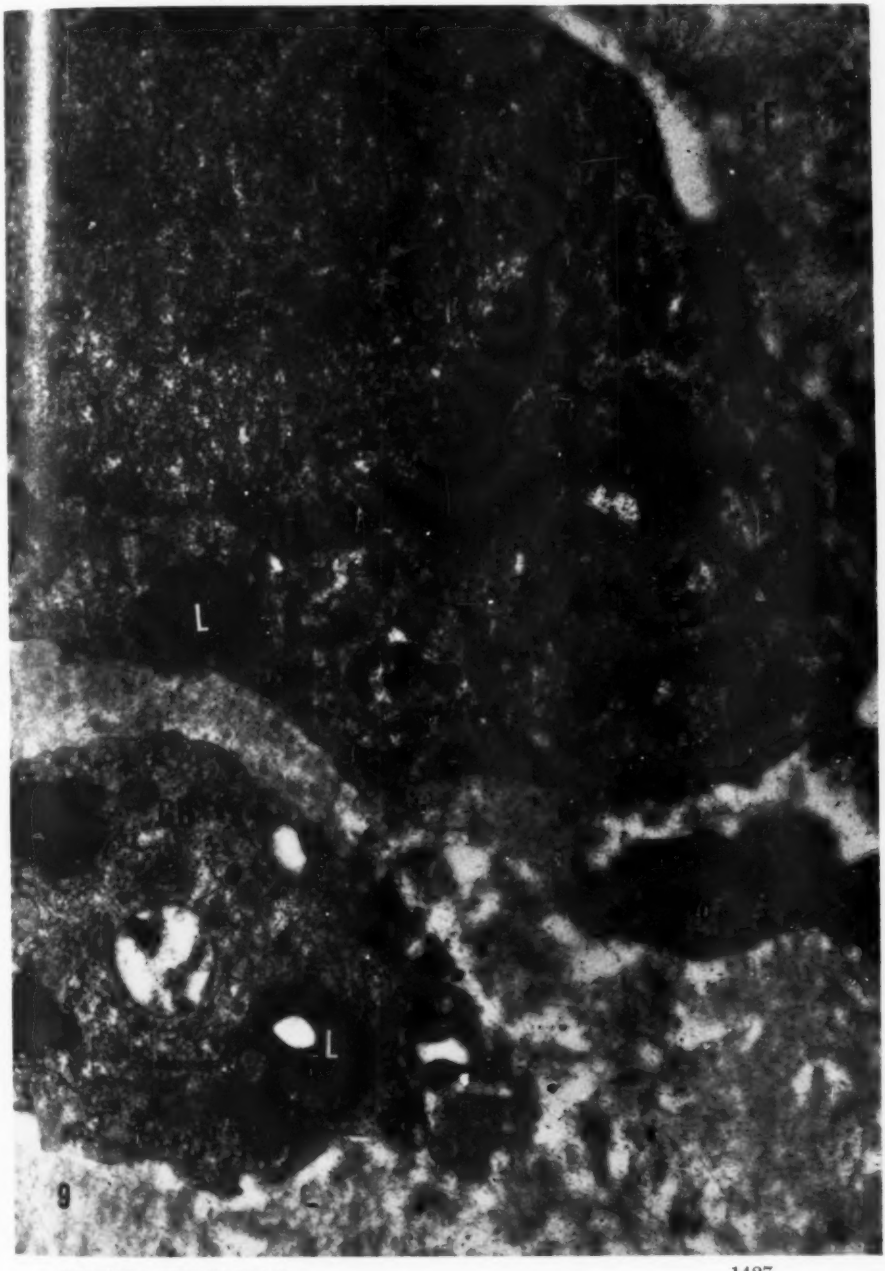

L
L
9

PLATE 201

FIGURE 10.—Large number of gray bodies, almost entirely filled with distinct virus particles, are observed in cytoplasm of chondrocyte. Membrane bounding gray bodies is indistinct. Few virus particles (VP) are observed free in cytoplasm. × 60,000

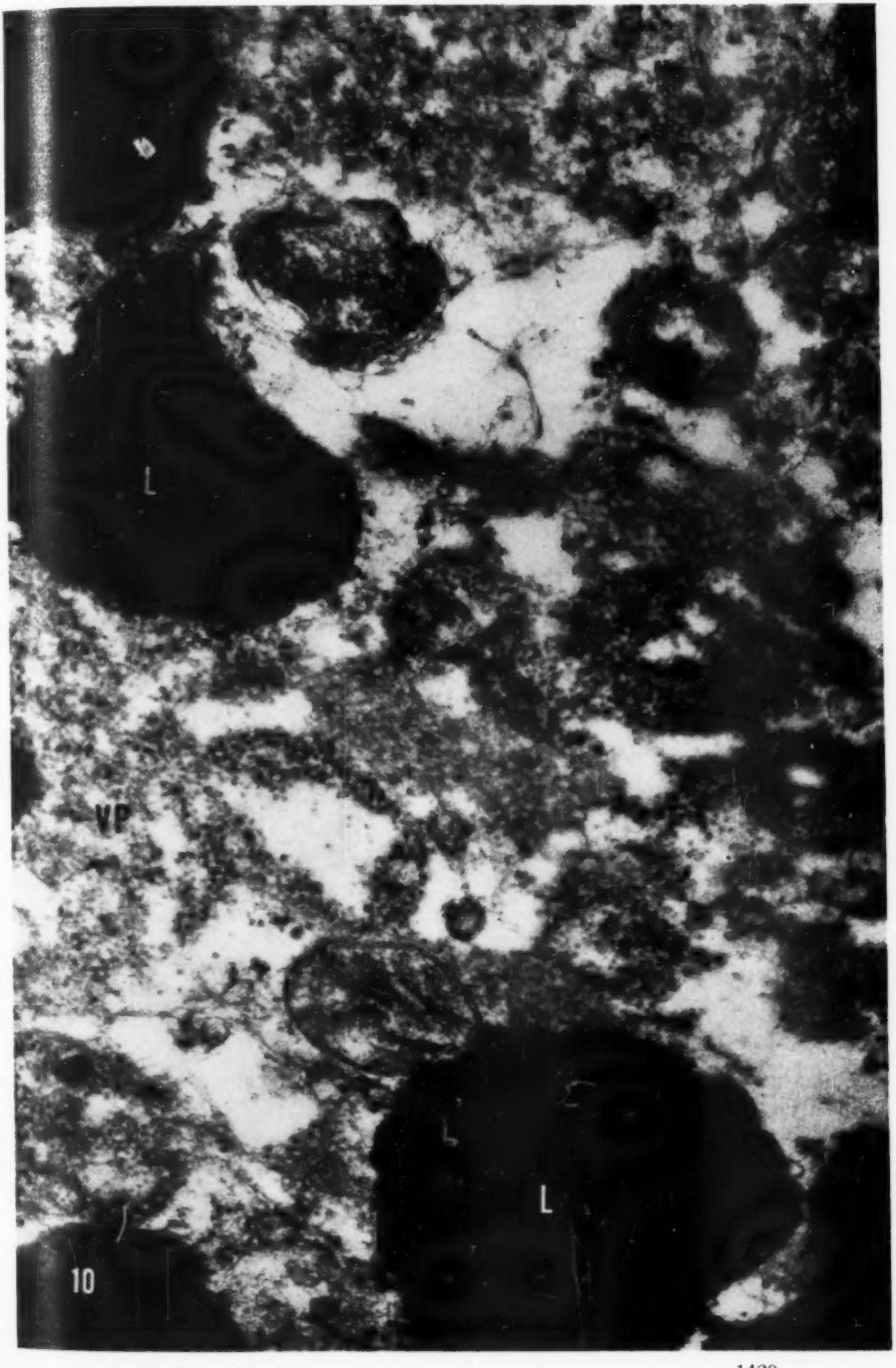

L
VP
L
10

PLATE 202

FIGURE 11.—Portion of very large gray body filled with virus particles. Particles are not arranged in crystalline pattern. In some instances, particle appears to be surrounded by 6 others. × 120,000

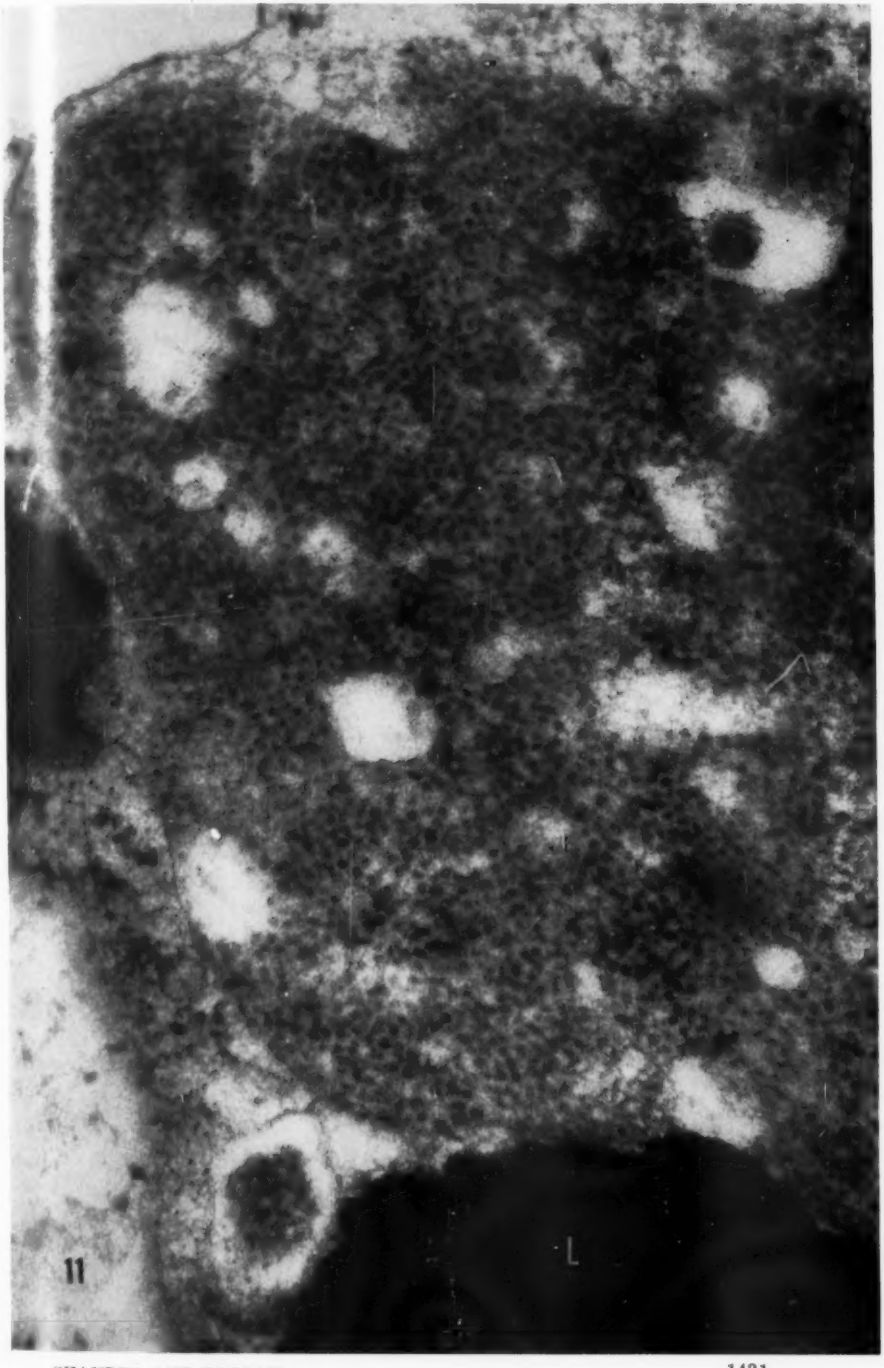

11
L

PLATE 203

FIGURE 12.—Portion of figure 11 at higher mangification. *Single arrow* points to row of particles which appear to be arranged next to one another (*see* text). *Double arrows* point to hexagonal patterns. × 400,000

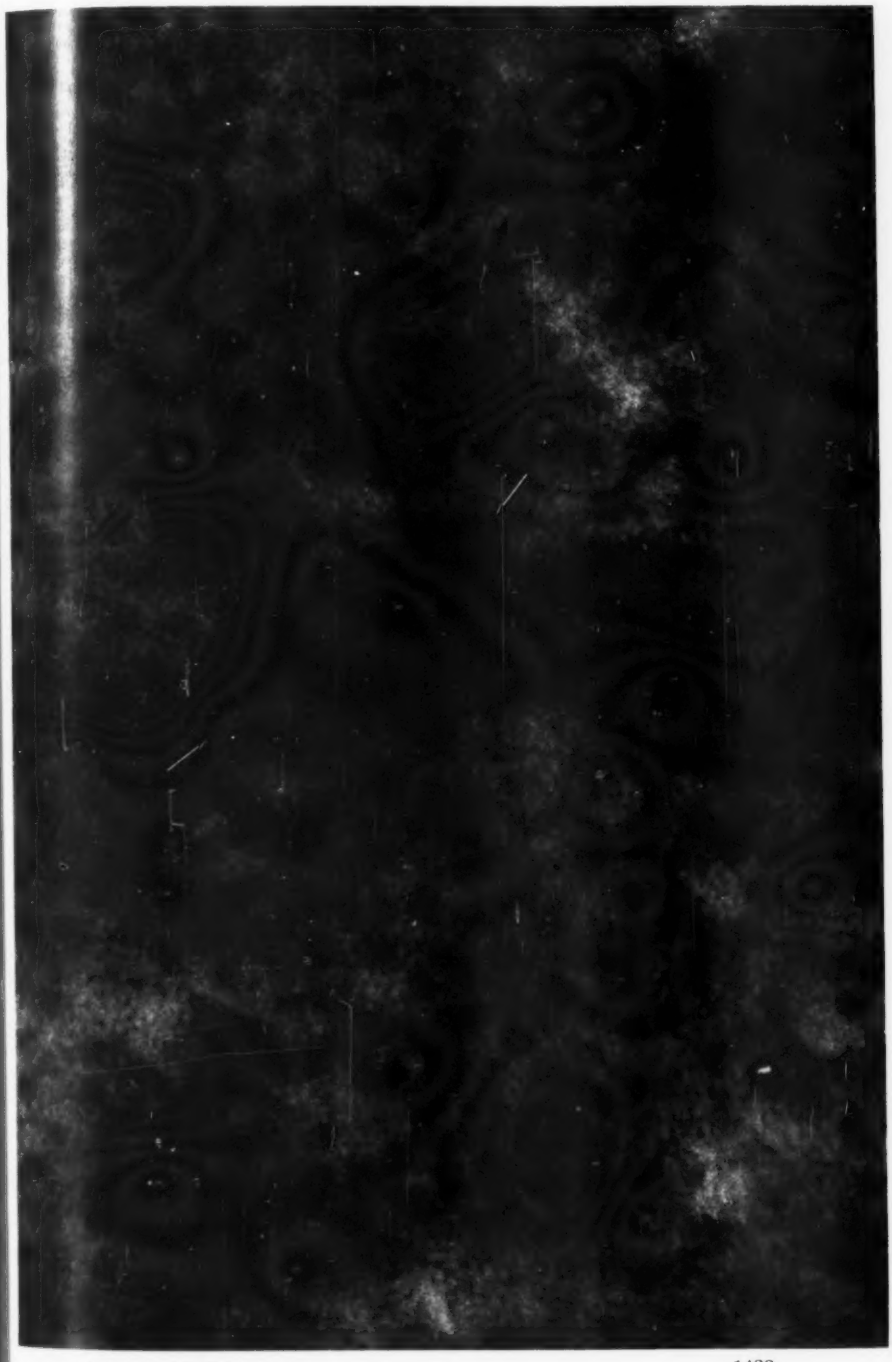


PLATE 204

FIGURE 13.—Cytoplasm of interstitial cell in kidney, which shows mitochondria (M) with regular organization of internal membrane, 1 altered mitochondrion (M3) (*see* text), and dilated profiles of endoplasmic reticulum. M3 could also be considered a gray body. Virus particles (VP) are visible within 3 bodies. Nucleus (N), in *lower region*, is sparsely filled with chromatin material. Particular appearance of cytoplasm of interstitial cell in this figure and also in figures 14 to 18 is not a result of poor preservation (*see* text). × 45,000

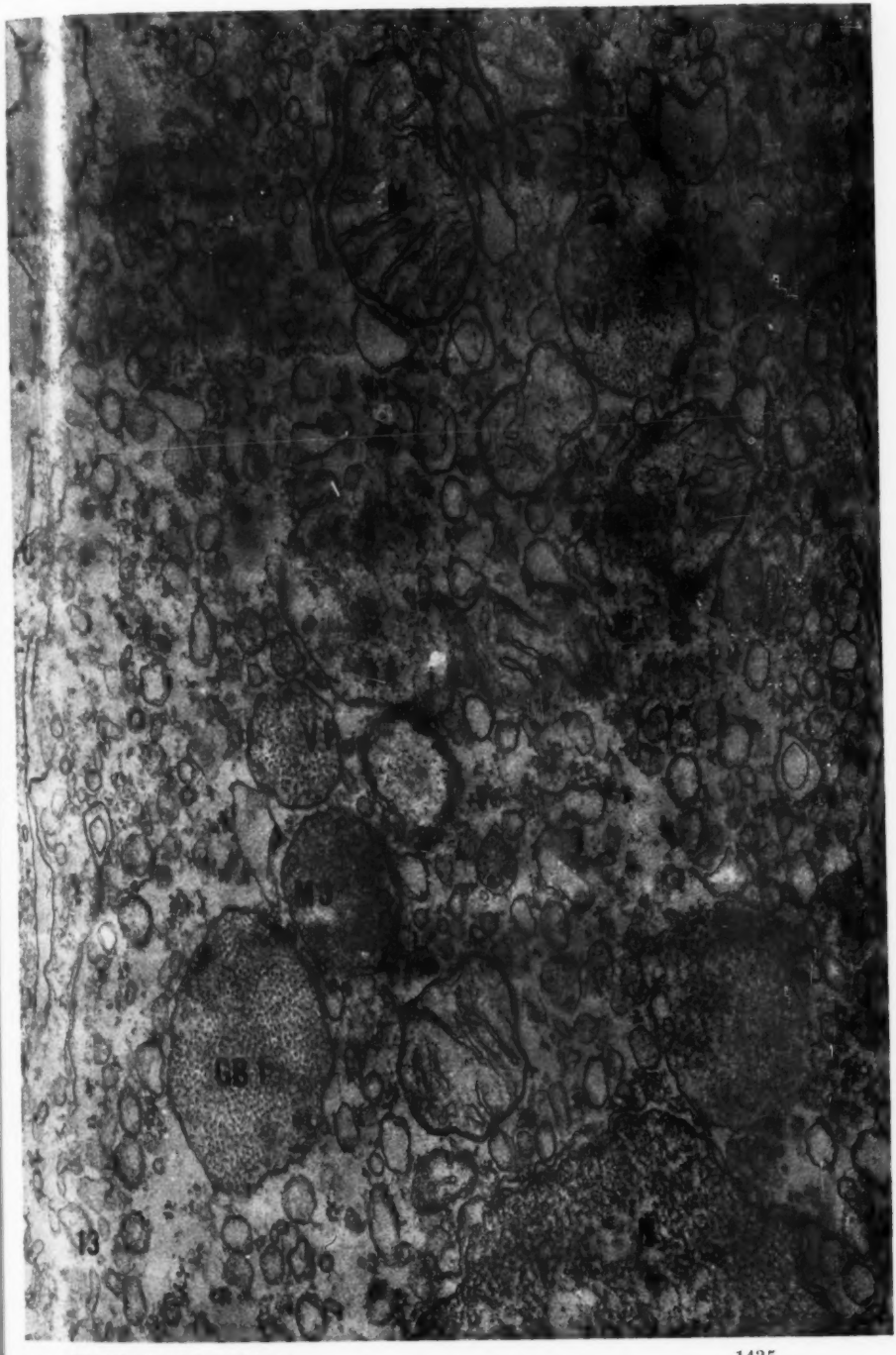



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PLATE 205

FIGURE 14.—Portions of 3 interstitial cells in kidney, which show altered mitochondria (M1 and M3). Outer nuclear membrane extends into cytoplasm to form elements of endoplasmic reticulum (*arrows*). One gray body contains virus particles (VP). Cell membrane is broken, probably as a result of injury to these cells during cutting of tissue. × 48,000

PLATE 206

FIGURE 15.—Portions of 2 interstitial cells in kidney. Gray body contains virus particles (VP). There are alterations in mitochondrial structure (M2 and M3). × 60,000

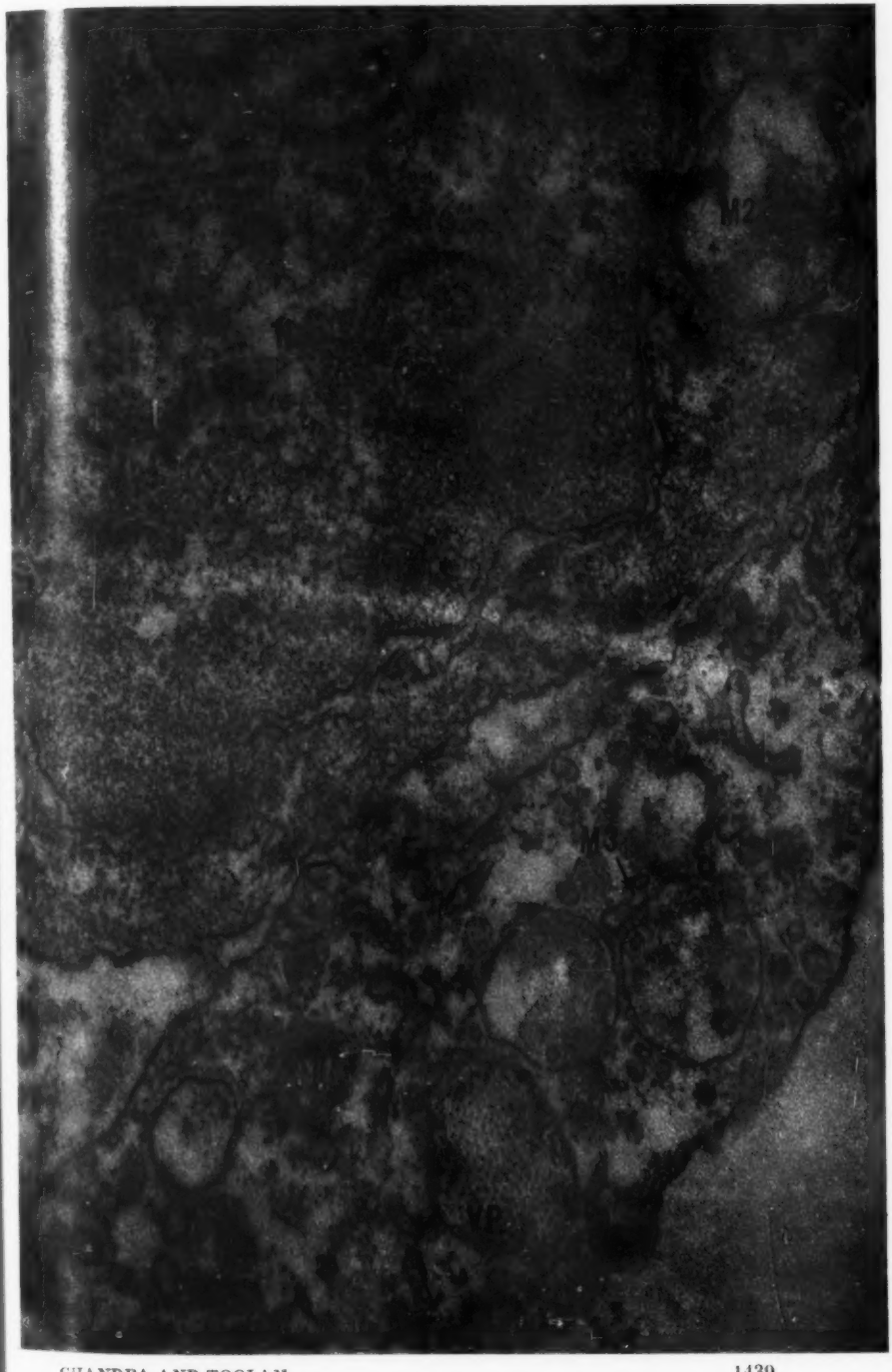

M2
M3
M2

PLATE 207

FIGURE 16.—Two altered mitochondria (M3) in what may be 2 interstitial cells. (*See* text for explanation of M3.) Major portion of surface of altered mitochondrion in *center* is bounded by double membrane. Particular appearance of inner membrane could be a result of its folding on itself. A few particles, similar in morphology to virus particles, are also seen in this altered mitochondrion. Basement membrane (BM) of tubule cell is seen in *right* portion of electron micrograph. × 60,000

FIGURE 17.—Body filled with virus particles at *lower right* and single membrane-bound body, probably altered mitochondrion (*see* text), on *upper left*. Latter body contains a few virus particles. × 120,000

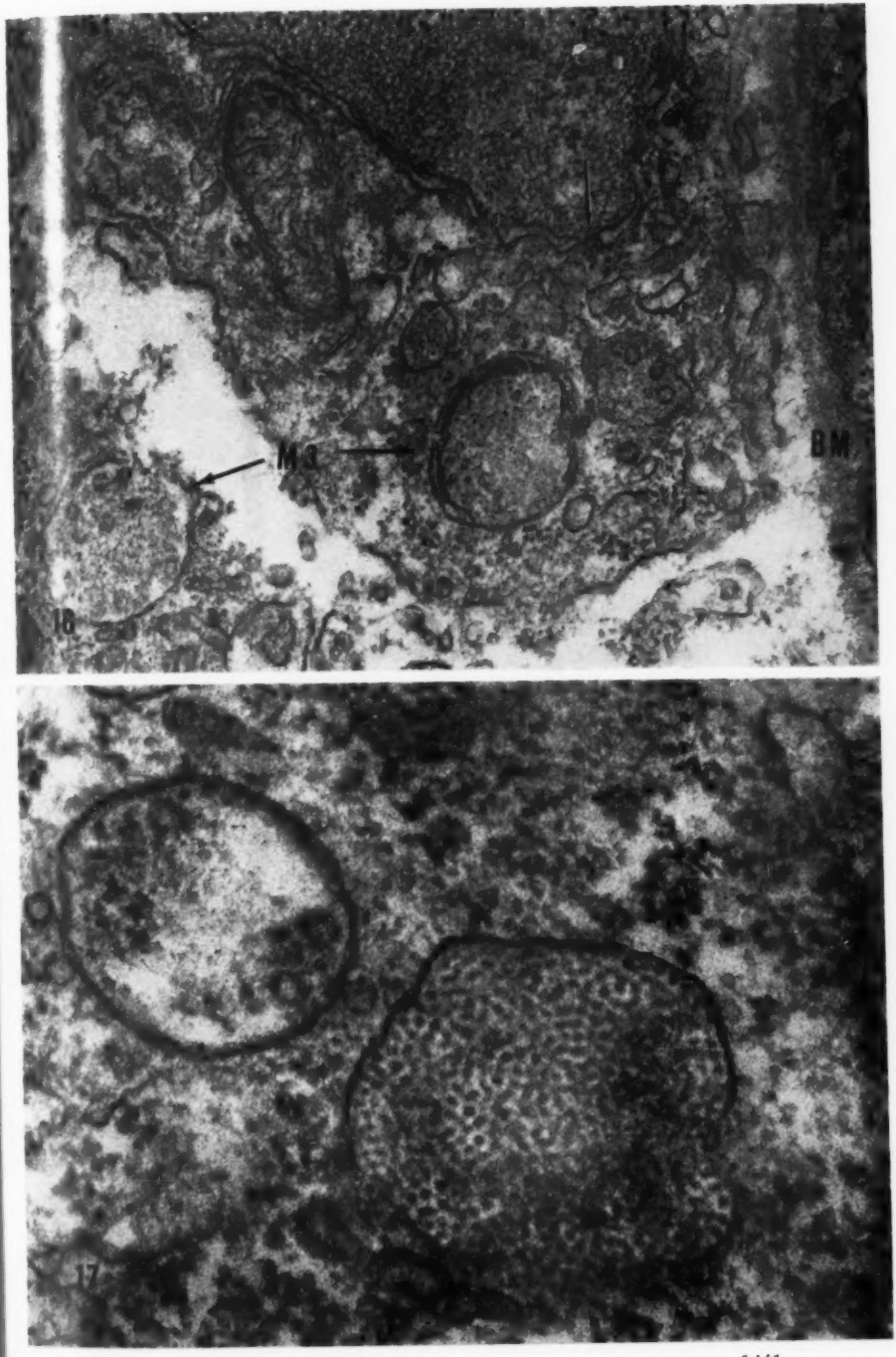
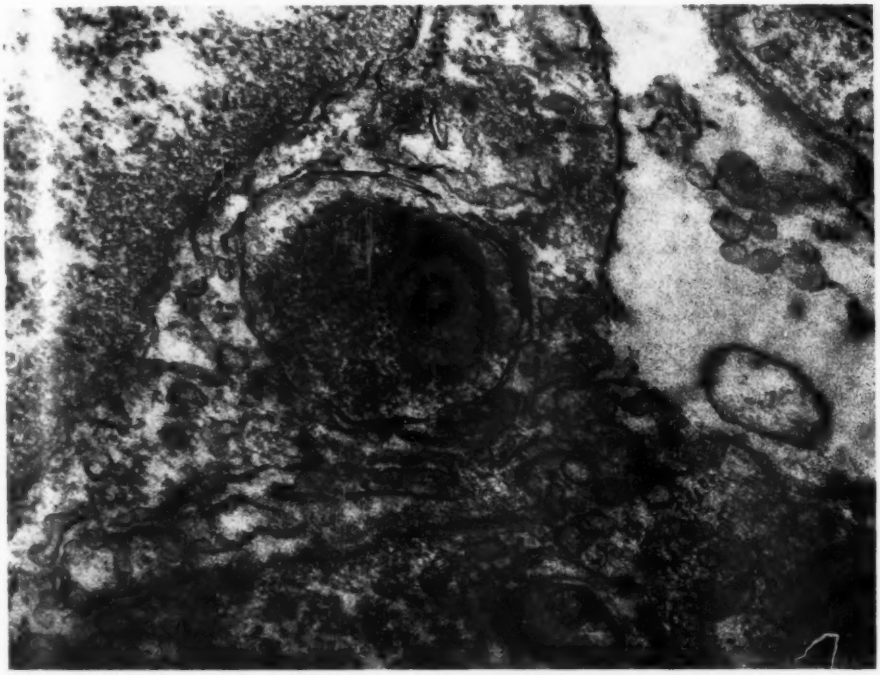

MS
BM
18
17

PLATE 208

FIGURE 18.—Cytoplasm of interstitial cell in *upper right* portion of electron micrograph contains a body bounded in a few areas by double membrane. Inside this body is profile of altered mitochondrion (M1) and large number of particles more regular in size and shape than RNP granules. × 60,000

FIGURE 19.—Body, bounded by double membrane and containing dense particles and mitochondrial profiles, is observed in cytoplasm of tubule cell of kidney. Other mitochondria (M1) in electron micrograph show indistinct cristae that could represent either tangential cut or alteration in mitochondrial structure as result of viral infection. × 60,000



M1
M1
19

PLATE 209

FIGURE 20.—Portion of cytoplasm of tubule cell in kidney. Body marked "1" is bounded by double membrane and contains complete profile of mitochondrion. Some granules, similar to RNP granules, occupy rest of space in body "1." Body marked "2" contains 2 altered mitochondria. *Arrow* points to large extension of outer membrane of an altered mitochondrion. Body marked "3" may be a later stage than that of body marked "2." × 48,000

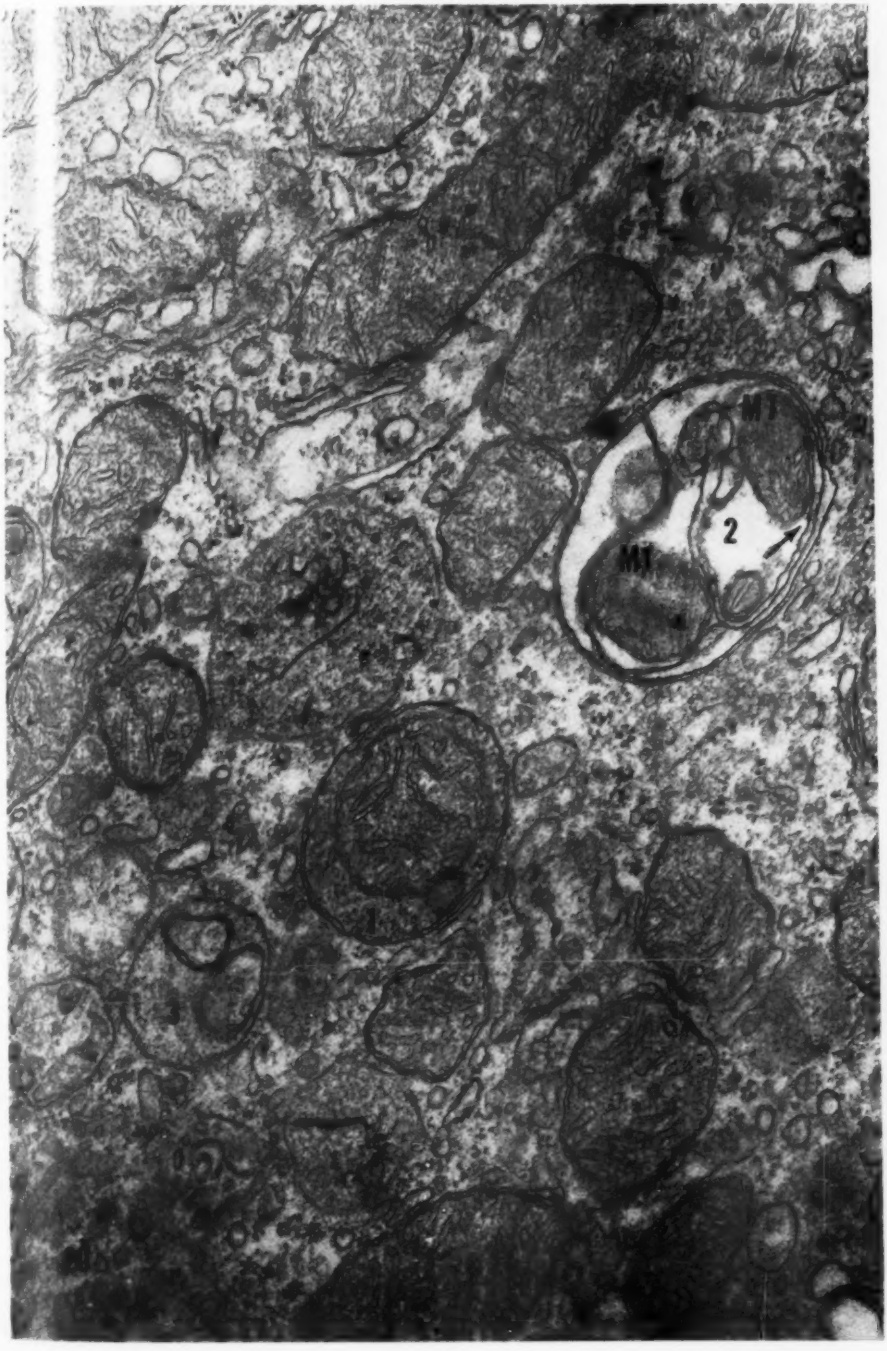
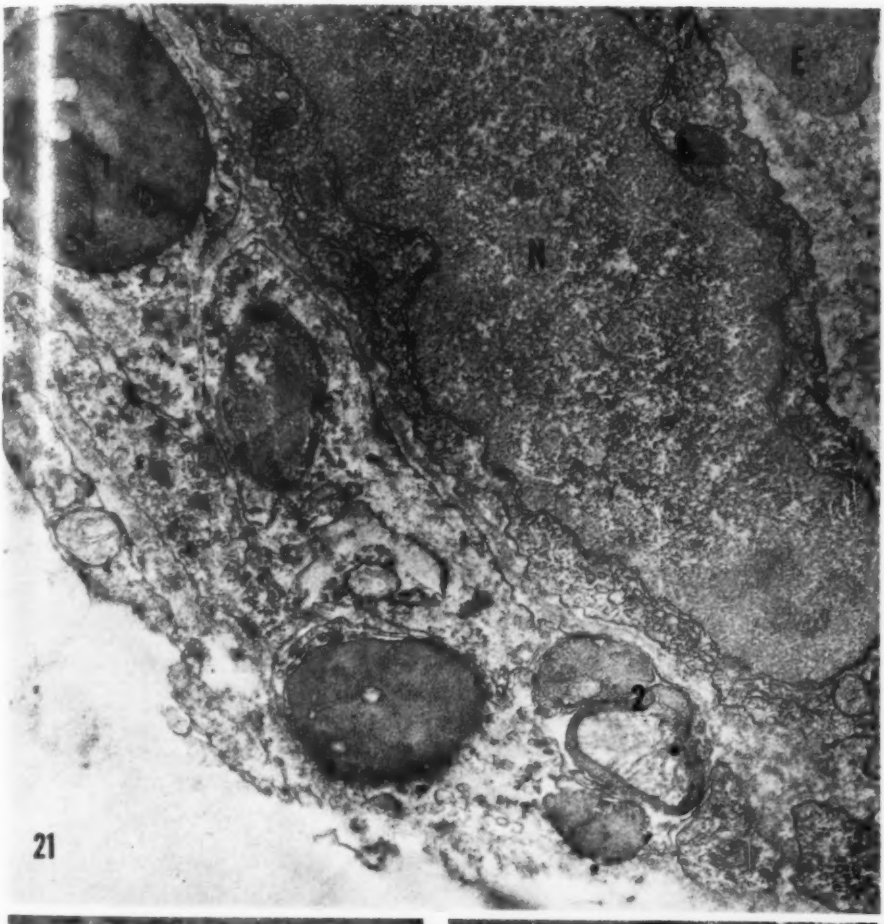

M1
2
M1

PLATE 210

FIGURE 21.—Interstitial cell in kidney containing 4 dense bodies, each bounded essentially by single membrane. Two of these bodies contain profiles of mitochondria. Segment of endothelial cell runs almost diagonally in *upper right* area of electron micrograph. Portion of erythrocyte (E) is also seen. × 30,000

FIGURE 22.—Small area of dense body in *center* in figure 21 at higher magnification. Particles measuring 150 A are distinctly seen. × 120,000

FIGURE 23.—Small portion of dense body in *upper left* corner in figure 21 at higher magnification. Mitochondrial structure is clearly seen. Matrix of mitochondrion appears to fuse with that of dense body. Particles are seen inside body but not within mitochondrion. × 60,000


E
N
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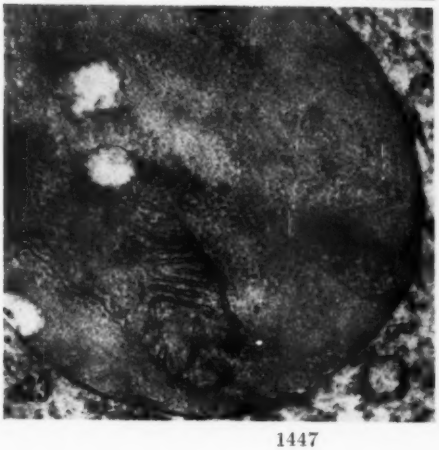
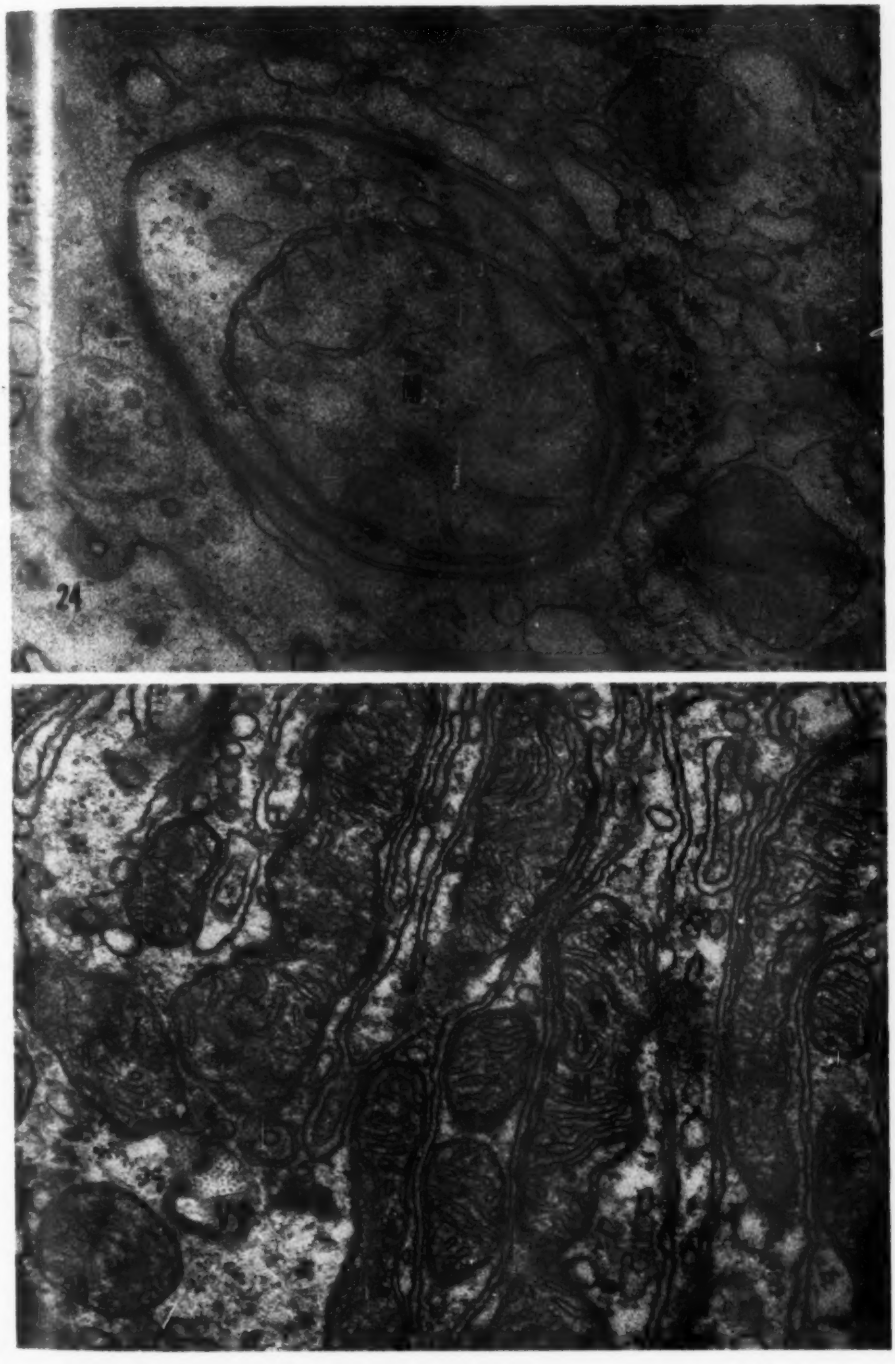


PLATE 211

FIGURE 24.—Hepatic cell mitochondrion enclosed inside double-membrane body. It is not clear whether this double membrane is part of plasma membranes of 2 adjacent cells. × 60,000

FIGURE 25.—Portion of cytoplasm of convoluted tubule cell of kidney which shows elongated mitochondria and infoldings of plasma membrane. Virus particles are distinctly seen. × 48,000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# New Metabolites of *N*-3-Fluorenylacetamide [1,2]

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SUMMARY

**Two new metabolites, *N*-(7-hydroxy-3-fluorenyl)acetamide and *N*-(2,7-dihydroxy - 3 - fluorenyl)acetamide, were identified in the urine of rats receiving an injection of *N*-3-fluorenylacetamide. These compounds were present chiefly as glucuronic acid conjugates, but were also excreted as sulfates. *N*-(7-Hydroxy-3-fluorenyl)acetamide was made from 7-methoxy-2-fluorenamine by an eight-step synthesis, while *N*-(2,7-dihydroxy-3 - fluorenyl)acetamide was prepared from the same starting material in six steps.—J. Nat. Cancer Inst. 27: 1451-1463, 1961.**

PREVIOUS WORK in this laboratory has shown that *N*-(2-hydroxy-3-fluorenyl)acetamide (2-OH-3-FAA) is a metabolite (*1*) of the weak carcinogen *N*-3-fluorenylacetamide (3-FAA) (*2*). It appeared reasonable that *N*-(7-hydroxy-3-fluorenyl)acetamide (7-OH-3-FAA) could also be formed during the metabolism of 3-FAA. Further research (*3*) has suggested the production of dihydroxylated derivatives during the metabolism of the strong carcinogen *N*-2-fluorenylacetamide (2-FAA). It therefore seemed probable that a dihydroxylated compound, such as *N*-(2,7-dihydroxy-3-fluorenyl)acetamide (2,7-diOH-3-FAA), might be derived from 3-FAA. The present report deals with the chemical syntheses of these new compounds and with the demonstration of their occurrence in the urine of rats administered 3-FAA.

## MATERIALS AND METHODS

### Syntheses of New Compounds

*General comments.*—The syntheses of these new metabolites started with 7-methoxy-2-fluorenamine (*4*). This compound was converted to 7-

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[3] This research was performed in the Laboratory of Biochemistry, National Cancer Institute.

[4] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.



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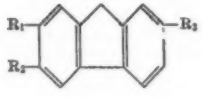
methoxy-2-fluorenol (I). Acetylation, followed by nitration under carefully controlled conditions to avoid disubstitution, gave 7-methoxy-6-nitro-2-acetoxyfluorene (III). To prove that the nitro group was indeed *ortho* to the methoxy group in III, the compound was reduced and acetylated to yield 6-acetamido-7-methoxy-2-acetoxyfluorene (V). Hydrolysis of III in ethanol-hydrochloric acid afforded 7-methoxy-6-nitro-2-fluorenol (VI). Reduction of VI and exhaustive acetylation yielded only V and no oxazole, which thus proved that the nitro group had not entered the ring bearing the acetoxy grouping. These conclusions were confirmed by the infrared spectra of the compounds. Hydrolysis of III and demethylation with hydrobromic acid, reduction of the resulting 3-nitro-2,7-fluorenediol (VIII), and acetylation without isolation of the unstable intermediate, 3-amino-2,7-fluorenediol, yielded 2,7-diOH-3-FAA (IX).




| | $R^1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| I | $OCH_3$ | H | OH |
| II | $OCH_3$ | H | $OCOCH_3$ |
| III | $OCH_3$ | $NO_2$ | $OCOCH_3$ |
| IV | $OCH_3$ | $NH_2$ | $OCOCH_3$ |
| V | $OCH_3$ | $NHCOCH_3$ | $OCOCH_3$ |
| VI | $OCH_3$ | $NO_2$ | OH |
| VII | $OCH_3$ | $NH_2$ | OH |
| VIII | OH | $NO_2$ | OH |
| IX | OH | $NHCOCH_3$ | OH |
| X | $NH_2$ | $NO_2$ | $OCH_3$ |
| XI | H | $NO_2$ | $OCH_3$ |
| XII | H | $NO_2$ | OH |
| XIII | H | $NHCOCH_3$ | OH |

The synthesis of 7-OH-3-FAA also began with 7-methoxy-2-fluorenamine which was acetylated and nitrated to yield 7-methoxy-3-nitro-2-fluorenamine (X) (*5*). Diazotization and removal of the amino group was accomplished, though in low yield, by the procedure of Kloetzel *et al.* (*6*). Demethylation afforded XII which after reduction and acetylation gave 7-OH-3-FAA (XIII).

As was noted in a previous paper dealing with 2,6-(3,7-) disubstituted fluorenes (*7*), the ultraviolet spectra of many of these compounds exhibit a characteristic arrangement of triple peaks. The spectra of 7-OH-3-FAA and 2,7-diOH-3-FAA, as well as 7-methoxy-6-amino- and -6-acetamido-2-acetoxyfluorene (IV and V), showed this type of configuration, thus providing additional examples and extending these correlations.

*Specific details.*—Melting points were usually determined in a capillary tube and are uncorrected. Infrared spectra were determined by P. H. Grantham on a Perkin-Elmer spectrophotometer as potassium bromide discs and ultraviolet spectra as $5 \times 10^{-5}$ molar solutions in ethanol on a Cary recording spectrophotometer. Microanalyses were done by the

staff of the Microanalytical Laboratory of the National Institutes of Health.

*7-Methoxy-2-fluorenol* (*I*).—7-Methoxy-2-fluorenamine (12.5 g) was dissolved in 1,200 ml of hot water and 80 ml of 6 N hydrochloric acid. After the solution was filtered and cooled to 5 to 10° C, 4.75 g of sodium nitrite in 10 ml of water was added with stirring. The diazonium solution was stirred for 50 minutes and then added over a period of 70 minutes to 850 ml of refluxing 4 N sulfuric acid. After standing overnight the precipitate was filtered off, dissolved in 1 liter of water containing 15 g of KOH, filtered, and reprecipitated. There was obtained 10 g of gray material, softening at 168°, mp 174°. Three or four crystallizations from ethanol-water gave the analytical sample which melted at 183–184°. $\lambda_{max}^{EtOH}$ 276 mμ (ε = 26,700); 317 (5,310); shoulder at 327 (5,010); $\lambda_{min}$ 237 (2,140); 303 (3,700). *Anal.* Calcd. for $C_{14}H_{12}O_2$: C, 79.22; H, 5.70. Found: C, 79.01; H, 5.84.

*7-Methoxy-2-acetoxyfluorene* (*II*).—Acetylation of the fluorenol with acetic anhydride gave 93 percent of crude material, mp 108°. Chromatography of a benzene solution on alumina followed by crystallization from dilute ethanol, benzene-petroleum ether, and cyclohexane gave white crystals, mp 115–116°, in a 75 percent yield. $\lambda_{max}^{EtOH}$ 275 mμ (ε = 23,100); 306 (6,800); 316.5 (6,560); $\lambda_{min}$ 236 (1,990); 300 (5,900); 312 (6,260). *Anal.* Calcd. for $C_{16}H_{14}O_3$: C, 75.57; H, 5.55. Found: C, 75.63; H, 5.65.

*7-Methoxy-6-nitro-2-acetoxyfluorene* (*III*).—To a well-stirred solution of 3.7 g of 7-methoxy-2-acetoxyfluorene in 25 ml of glacial acetic acid at 15 to 20°, there was added dropwise a mixture of 3.5 ml of concentrated nitric acid and 3.5 ml of water. The temperature rose to 25° and a thick, pasty mass formed. Stirring was continued for 0.5 hour. After addition of water, 4.4 g of yellow material, mp 185–188°, was obtained. The benzene solution was percolated through a 1 × 11 cm column of Woelm neutral alumina, and the eluate was evaporated to give 3.9 g of fluffy, bright-yellow needles, mp 193–195°. Further crystallization from benzene gave an analytical sample, mp 197–198°. $\lambda_{max}^{EtOH}$ 265 mμ (ε = 28,100); 296 (11,970); 360 (2,470); $\lambda_{min}$ 231.5 (10,530); 294 (11,830); 327 (1,760). *Anal.* Calcd. for $C_{16}H_{13}NO_5$: C, 64.21; H, 4.38; N, 4.68. Found: C, 64.03; H, 4.42; N, 4.44.

Catalytic low pressure hydrogenation of 1.5 g of III with 150 mg of platinum oxide in 150 ml of ethanol yielded 1.3 g of crude *7-methoxy-6-amino-2-acetoxyfluorene* (IV), which melted at 192–193.5°, after crystallization from ethanol. $\lambda_{max}^{EtOH}$ 240 mμ (ε = 22,800); 278 (13,660); 329 (12,270); $\lambda_{min}$ 225 (18,530); 261 (8,900); trough 293–301 (5,600). *Anal.* Calcd. for $C_{16}H_{15}NO_3$: C, 71.36; H, 5.61. Found: C, 71.25; H, 5.45.

Refluxing this amine with acetic anhydride in benzene for 0.5 hour yielded white needles of *6-acetamido-7-methoxy-2-acetoxyfluorene* (V) mp 245°, after crystallization from ethanol-water or benzene. The infrared spectrum showed carbonyl bands at 5.72 and 5.88 μ and a strong band at 8 to 8.5 μ (acetate ester). $\lambda_{max}^{EtOH}$ 244 mμ (ε = 25,450); 276 (22,950); 326

(10,600); shoulders at 251 (24,550) and 315 (9,880); $\lambda_{min}$ 229 (18,860); 261 (17,360); 303 (6,590). *Anal.* Calcd. for $C_{18}H_{17}NO_4$: C, 69.44; H, 5.51. Found: C, 69.57; H, 5.99.

Hydrolysis of 3.4 g of III in 100 ml of ethanol and 20 ml of 6 N hydrochloric acid yielded 2.4 g of *7-methoxy-6-nitro-2-fluorenol* (VI), mp 232–233°. $\lambda_{max}^{EtOH}$ 272 mμ (ε = 27,320); 380 (1,970); shoulder 309 (10,670); $\lambda_{min}$ 235 (8,650); 340 (1,400). *Anal.* Calcd. for $C_{14}H_{11}NO_4$: C, 65.36; H, 4.31. Found: C, 65.01; H, 4.63.

*6-Amino-7-methoxy-2-fluorenol* (*VII*).—Catalytic reduction of 1.7 g of VI yielded 1.2 g of crude amine, mp 232°. Three crystallizations of 0.7 g from ethanol-water yielded 0.375 g, mp 241° (dec). $\lambda_{max}^{EtOH}$ 241.5 mμ (ε = 18,200); 280 (17,140); 298.5 (7,650); 335 (11,220). $\lambda_{min}$ 232 (16,800); 260 (9,530); 296.5 (7,580); 310 (6,040). *Anal.* Calcd. for $C_{14}H_{13}NO_2$: C, 73.99; H, 5.76. Found: C, 74.17; H, 5.75.

Refluxing 0.5 g of VII with 5 ml of acetic anhydride and 1.2 g of fused sodium acetate for 7 hours, followed by chromatography of a benzene solution of the product on Merck alumina and crystallization, yielded 0.26 g of *6-acetamido-7-methoxy-2-acetoxyfluorene* (V), as proved by mp, mixture mp, and infrared spectrum.

Refluxing 3 g of III in 45 ml of glacial acetic acid and 45 ml of 48 percent hydrobromic acid for 4 hours yielded 3 g of bright-red crystals, mp 192–195°. This was crystallized twice from xylene and three times from 50 percent aqueous ethanol to yield 0.9 g of red *3-nitro-2,7-fluorenediol* (VIII), mp 215–216°. $\lambda_{max}^{EtOH}$ 277 mμ (ε = 30,700); 435 (2,850); $\lambda_{min}$ 235 (6,240); 360 (840). *Anal.* Calcd. for $C_{13}H_9NO_4$: C, 64.16; H, 3.73; N, 5.76. Found: C, 64.05; H, 3.98; N, 5.63.

N-(*2,7-Dihydroxy-3-fluorenyl*)*acetamide* (*IX*).—VIII (0.5 g) was hydrogenated with platinum oxide catalyst in ethanol. The amine solution obtained was filtered into dilute hydrochloric acid in an atmosphere of sulfur dioxide to prevent oxidation. Otherwise a deep-blue solution formed very quickly. Evaporation under a nitrogen-sulfur dioxide atmosphere gave a white residue. This was dissolved in 50 ml of hot water and 1 ml of 6 N hydrochloric acid, filtered, cooled, the *p*H adjusted to 5 to 6 with 6 g of sodium acetate, and the amine was acetylated by stirring in the cold with 6 ml of acetic anhydride for 5 to 6 hours to give 0.5 g of product, mp 229–230° (dec). The analytical sample, obtained after crystallization from 50 percent aqueous acetic acid or ethanol had mp 229.5–230° (dec). $\lambda_{max}^{EtOH}$ 242.5 mμ (ε = 21,740); 278 (22,730); 331 (8,710); $\lambda_{min}$ 232 (17,750); 261 (14,860); 306 (3,890). *Anal.* Calcd. for $C_{15}H_{13}NO_3$: C, 70.57; H, 5.13. Found: C, 70.21; H, 5.05.

*6-Nitro-2-methoxyfluorene* (*XI*).—A solution of 1 ml of water and 29 ml of concentrated sulfuric acid was cooled with stirring in an ice-salt bath to −2°. Powdered sodium nitrite (0.4 g) was added in small portions and then 1.1 g of powdered 7-methoxy-3-nitro-2-fluorenamine (X) over a period of 15 minutes. After the diazonium solution was stirred in the cold for 1 hour, 35 ml of cold hypophosphorous acid was added dropwise over a period of 1 hour, with the temperature maintained at −5 to 0°.

The reddish mixture was kept at −10° for 5 to 6 days and then at 4° for 2 days. The mixture was poured on ice; the red-brown precipitate was filtered off, dried, and extracted repeatedly with benzene. The extract was percolated through an alumina column. From the pale-yellow eluate there was obtained 0.15 to 0.3 g of yellowish crystals (in various runs), softening at 130°, mp 133–135°. Crystallization from acetone or ethanol yielded needles, mp 138–140°. $\lambda_{max}^{EtOH}$ 269 mμ (ε = 30,900); 303.5 (15,150); 350 (1,300); $\lambda_{min}$ 237 (7,740); 301.5 (14,900); 335 (1,260); shoulder at 292.5 (17,240). *Anal.* Calcd. for $C_{14}H_{11}NO_2$: C, 69.70; H, 4.60. Found: C, 69.58; H, 4.63.

*6-Nitro-2-fluorenol* (*XII*).—A solution of 0.1 g of XI in 6 ml of acetic acid and 6 ml of 48 percent hydrobromic acid was refluxed for 2 hours. Addition of ice yielded 0.1 g of crude material, mp 192–195°. A benzene solution was percolated through Woelm neutral alumina. The eluate was taken to dryness and the residue was crystallized 5 times from benzene-petroleum ether and acetone-water to yield 45 mg of yellow needles of 6-nitro-2-fluorenol, mp 210.5–212°. $\lambda_{max}^{EtOH}$ 271 mμ (ε = 25,680); $\lambda_{min}$ 242 (8,470). *Anal.* Calcd. for $C_{13}H_9NO_3 \cdot H_2O$: C, 63.67; H, 4.52. Found: C, 64.10; H, 4.30. After sublimation at 800 μ and 150–160° the mp was 210.5–211°, but the water of crystallization was not eliminated (analysis C, 63.68; H, 4.49). On a Kofler block it was apparent that a temperature of at least 195° was necessary to remove the water of crystallization.

N-(*7-Hydroxy-3-fluorenyl*)*acetamide* (*XIII*).—Hydrogenation of 70 mg of XII with platinum oxide catalyst in ethanol followed by acetylation of the crude amine in aqueous acetate buffer yielded 73 mg of crude hydroxyacetamide, mp 245° (dec). Several crystallizations from ethanol-water afforded 23 mg of small white needles, mp 244–245° (Kofler). $\lambda_{max}^{EtOH}$ 215 mμ (ε = 27,790); 252 (28,050); 277 (17,120); 322.5 (9,350); $\lambda_{min}$ 232 (15,760); 266.5 (16,220); 302 (5,530); shoulder at 310.5 (7,470). *Anal.* Calcd. for $C_{15}H_{13}NO_2 \cdot \frac{1}{2} H_2O$: C, 72.56; H, 5.68. Found: C, 72.49; H, 5.96.

## METABOLISM STUDIES[5]

Each of 2 female Buffalo strain rats received an intraperitoneal injection of a suspension of 19 mg, 10 mg per 100 g body weight, of 3-FAA (a total of 170 μmoles) in a 7 percent gum acacia-isotonic saline solution. The rats were kept in metabolism cages and fed a semisynthetic 16 percent casein diet.[6] Controls received the casein diet alone. Urine, 34 ml, was collected in ice-cold receivers for 48 hours.

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[5] The animals used were kindly furnished by Dr. H. P. Morris. The assistance of Mr. P. H. Grantham in the metabolism experiments is gratefully acknowledged.

[6] I am indebted to Dr. H. Sidransky for kindly supplying the diet. Its composition was: casein 16, corn oil 5, sucrose 70, salt mix 4, and vitamin mix 5 percent, respectively. The vitamin mixture contained thiamine hydrochloride, 500 mg; riboflavine, 187.5 mg; pyridoxine hydrochloride, 500 mg; nicotinic acid, 1.875 g; calcium pantothenate, 1.3 g; 2-methyl-1,4-naphthoquinone, 250 mg; folic acid, 50 mg; vitamin $B_{12}$, 5 mg; biotin, 10 mg; choline chloride, 15 g; and oleum percomorphum, 50 ml (45.4 g).

Part of the urine, 23 ml (*see* tables 1 and 2), was adjusted to *p*H 6 with 2 ml of 1 M acetate buffer and extracted 5 times with 23 ml of ether (*AE*). The aqueous phase (*AA*) was incubated with 75 mg of bacterial β-glucuronidase (Sigma Chemical Co., St. Louis, Mo.) overnight, in the presence of 0.3 ml of chloroform. This solution was again extracted 5 times with ether (*AGE*) to remove any material freed by the enzymic hydrolysis. The aqueous phase (*AGA*) was acidified to *p*H 1 with 0.7 ml of 12 N HCl and was heated to boiling for 10 minutes to hydrolyze sulfuric acid esters, neutralized, and extracted with ether (*ASE*).

TABLE 1.—Determination of chromophoric material in the urine* and extracts of urine

| Fraction | Chromophoric material | | |
|---|---|---|---|
| | μmoles/ 10 ml† | Percent of total | Percent of fraction |
| Total urine (*U*) | 17 | 100 | |
| Ether extract (*AE*) | 0.74 | 4.4 | 5 |
| Aqueous portion (*AA*) | 14 | 81 | |
| (*AA*) after treatment with glucuronidase (glucuronides) | | | |
| Ether extract (*AGE*) | 7 | 41 | 64 |
| Aqueous portion (*AGA*) | 3.9 | 23 | |
| *AGA* after acid hydrolysis (sulfates) | | | |
| Ether extract (*ASE*) | 1.6 | 9.2 | 47 |
| Aqueous portion (*ASA*) | 1.8 | 10.5 | |

*Experiments performed with 23 ml of urine (*see* text).
†Expressed in terms of amounts in 10 ml of urine to permit comparison with data in table 2.

To another 10 ml portion of urine 10 g of $(NH_4)_2SO_4$ and 10 ml of water were added. After four extractions with 20 ml portions of ether-ethanol (3:1), the organic phase was percolated through a column made from 20 g of neutral alumina to separate unconjugated materials, sulfuric acid conjugates, and glucosiduronic acids (*8*, *9*). Unconjugated metabolites were eluted by 100 ml of the ether-ethanol mixture (*I*). Sulfate conjugates were removed from the column by 200 ml of ethanol, followed by 200 ml of 50 percent aqueous ethanol (*II*). The addition of 200 ml of phosphate-citrate buffer, *p*H 6, eluted the glucosiduronic acids (*III*), table 2.

The organic solvent from fraction *I* was evaporated and the residue was re-extracted with ether. Portions of the ether extract were chromatographed on paper or analyzed for diazotizable amino groups as described later.

Fraction *II* was made to *p*H 6 and incubated overnight at 37° with Taka-diastase, followed by ether extraction. This extract was worked up as described for fraction *I*. The glucuronide portion, fraction *III*, was half-saturated with $(NH_4)_2SO_4$ and extracted with ether-ethanol (3:1). The extract was evaporated. The residue was dissolved in 0.2 M acetate buffer, *p*H 6, and incubated at 37° with 50 to 100 mg of β-

TABLE 2.—Alumina column separation of metabolites*

| Fraction | By colorimetric method | | | | By ultraviolet absorbance (ether extractable material) | | | |
|---|---|---|---|---|---|---|---|---|
| | Total (μmoles) | Percent of urine | Ether extract (μmoles) | Aqueous (μmoles) | 2,7-diOH-3-FAA (μmoles) | 7-OH-3-FAA (μmoles) | 2-OH-3-FAA† (μmoles) | 3-FA (μmoles) |
| Unconjugated metabolites (I) | 1. 6 | 9 | 0. 4 | 0. 5 | t‡ | 0. 3 | 0. 27 | t‡ |
| Sulfate conjugates (II) | 2. 9 | 17 | 0. 6‖ | 1. 4‖ | 0. 11 | 0. 4 | — | — |
| Glucuronide conjugates (III) | 13 | 74 | 4. 9§ | 5. 5§ | 0. 19 | 5. 0 | 3. 4 | — |

*Ten ml of rat urine containing an equivalent of 17 μmoles of 3-FAA were used for this separation.
†This compound cannot be determined by the colorimetric method.
‡Trace.
‖After hydrolysis by Taka-diastase.
§After hydrolysis by β-glucuronidase.

glucuronidase overnight and also extracted with ether. The extract was treated similarly to fractions *I* and *II*.

The amount of diazotizable material in each fraction from the alumina column or the extraction procedures (tables 1 and 2) was estimated by the colorimetric method of Westfall (*10*), as extended by Dyer (*11*). In all instances a correction was applied for any color developed by the corresponding fraction from a control urine of animals on the same diet but not treated with 3-FAA. The determinations were performed at 510 mμ, wavelength of maximum absorbance for the dye from diazotized 3-fluorenamine (3-FA) and R-salt. The color density followed Beer's law in the range of 0.06 to 0.48 μmole of 3-FA.

Aliquots of the various fractions and solutions of the standard compounds, 3-fluorenamine, 2-OH-3-FAA, 7-OH-3-FAA, and 2,7-diOH-3-FAA were applied on Whatman #1 paper and chromatographed in the cyclohexane-*tert*-butanol-acetic acid-water (16:4:2:1 parts) or *sec*-butanol-3 percent ammonia (3:1) solvent systems described previously (*12*). Some of the paper strips were sprayed with Folin-Ciocalteu's reagent or placed in an atmosphere of chlorine to develop the color. The corresponding regions on the untreated strips were cut out and eluted by 1 ml of ethanol. The ultraviolet-absorption curve of this solution was determined on a Cary recording spectrophotometer, and the concentration of each metabolite was determined by reference to the standard spectrum of the pure synthetic material.

## RESULTS

### Colorimetric Studies

In the 48-hour period 34 ml of urine was collected, which contained 57.6 μmoles of chromophoric material (34% of the dose), table 1. Two hydroxylated metabolites gave appreciable values in the diazo-R-salt test for 3-FAA; 2,7-diOH-3-FAA gave a greenish color which had an absorbance equivalent to 22 percent interference in the readings for 3-FAA. The other metabolite, 7-OH-3-FAA, gave a pink color which had an absorbance equivalent to 109 percent that from 3-FAA. However, at 510 mμ the third metabolite 2-OH-3-FAA gave no reading (*cf. also 11*) and thus could not contribute to the values obtained by the diazo-R-salt method.

Ether extraction of a urine sample transferred about 4 percent of the chromophoric material to the organic phase. This amount represented grossly the quantity of free metabolites. After treatment with bacterial β-glucuronidase, another 41 percent of the chromophoric material initially present (but 64% of the material in this particular step) was rendered ether-extractable. Further hydrolysis by very dilute acid usually used for splitting sulfuric acid esters permitted the extraction of another 9 percent to the ether layer. The results observed in the present instance

with *N*-3-fluorenylacetamide were of the same order of magnitude as those noted with *N*-2-fluorenylacetamide (*12*).

A more direct assay, yielding however a similar picture (table 2), of the relative amounts of unconjugated and conjugated metabolites was obtained by column chromatography on an alumina column (*8, 9*). Thus, the bulk of the chromophoric material was found in fraction *III* containing the glucosiduronic acids. One fourth that amount occurred in the eluate (*II*) containing sulfuric acid esters, and about half of this latter quantity was associated with the free compounds (*I*). Hydrolysis by the appropriate enzymes and ether-extraction experiments gave further evidence regarding the nature of the compounds so obtained. More than half the chromophoric material remained in the aqueous layer after glucuronidase hydrolysis; its constitution remains to be investigated. Each of the extracts was investigated by paper chromatography as discussed later.

## Chromatographic Studies

Chromatography of the standard compounds in the cyclohexane system gave spots which were fairly well separated (table 3). As might be expected, 2,7-diOH-3-FAA, being more polar and more water soluble, moved slowly, while 3-FA was the fastest. The other compounds, 2-OH-3-FAA and 7-OH-3-FAA, had intermediate $R_F$ values. In the butanol-solvent system, all the hydroxylated compounds had almost similar $R_F$ values of 0.91 at the front edge. In some cases, strips with the 2,7-diOH-3-FAA standard gave 2 spots. The slower moving of these may have been due to an ammonium salt of 2,7-diOH-3-FAA.

All the standard materials gave a bluish coloration with the Folin reagent. However, with 2,7-diOH-3-FAA, the color was at first dirty yellow and the blue developed more slowly. In addition, this compound gave a characteristic yellow with chlorine which was not observed with the other reference compounds. Chromatography of the ether extract (*AE*) of urine from rats given 3-FAA showed 3 spots in the cyclohexane system, the $R_F$ values of which corresponded well with those of the standards indicating that all three hydroxylated compounds were present as metabolites. In the butanol solvent 2 spots were noted. The first, $R_F$ 0.87–0.92, was due to the 3 hydroxylated metabolites. The spot $R_F$ 0.69–0.73 (footnote to table 3) may represent a still unknown metabolite. It was not visible on the chromatogram of the control urine.

The ether extract of glucuronidase-treated urine (fraction *AGE*) showed 3 spots in the cyclohexane system, corresponding to the 3 hydroxylated metabolites. In the butanol system 3 spots also appeared. The first 2 ($R_F$ 0.65–0.68 and 0.71–0.74) may have been caused by the same material with $R_F$ 0.69–0.73 that was present in fraction *AE*.

The ether extract containing sulfate-conjugated metabolites (fraction *ASE*) showed one very definite spot ($R_F$ 0.10–0.19) in the cyclohexane system, corresponding to 7-OH-3-FAA. A spot near the origin ($R_F$ 0.0–0.05) may have been due to a trace of 2,7-diOH-3-FAA. In the butanol system only 1 spot was seen.

TABLE 3.—$R_F$ values of standard compounds and metabolites

| Standards* | | | Ether-extractable metabolites (from table 1) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Unconjugated, fraction *AE* | | After hydrolysis of glucuronides, fraction *AGE* | | After hydrolysis of sulfates, fraction *ASE* | |
| Compound | Cyclohexane solvent | Butanol solvent | Cyclohexane | Butanol† | Cyclohexane | Butanol‡ | Cyclohexane | Butanol |
| *N*-(2,7-Dihydroxy-3-fluorenyl) acetamide | 0. 01–0. 08 | 0. 43–0. 60<br>0. 78–0. 91 | 0. 0–0. 03 | 0. 87–0. 92 | 0. 01–0. 03 | 0. 85–0. 91 | 0. 0–0. 05 | 0. 87–0. 91 |
| *N*-(7-Hydroxy-3-fluorenyl) acetamide | 0. 09–0. 19 | 0. 86–0. 93 | 0. 11–0. 18 | | 0. 09–0. 24 | | 0. 10–0. 19 | |
| *N*-(2-Hydroxy-3-fluorenyl) acetamide | 0. 46–0. 67 | 0. 85–0. 91 | 0. 48–0. 71 | | 0. 51–0. 74 | | — | — |
| 3-Fluorenamine | 0. 89–0. 94 | 0. 97–1. 0 | — | — | — | — | — | — |

*3-FAA does not give a test with the Folin reagent; all other compounds yield a blue color with this reagent; 2,7-diOH-3-FAA shows a yellow color on paper when exposed to chlorine.
†A spot with $R_F$ 0.69–0.73 was also noted.
‡Two additional spots with $R_F$ values of 0.65–0.68 and 0.71–0.74 were also observed.

Urine from the control rats was subjected simultaneously to the same procedures. Ether extracts of the control urine and of control urine after glucuronidase treatment showed no spots when chromatographed in the cyclohexane or butanol systems. The ether extract of acid-hydrolyzed urine (*ASE*) from both controls and experimental animals exhibited a spot with an $R_f$ value of 0.33–0.38 in the cyclohexane system. This spot fluoresced under ultraviolet light but did not react with the Folin reagent. Likewise in the butanol solvent, *ASE* from both controls and experimental animals gave a yellow fluorescence at $R_f$ 0.85–0.90, which was Folin-negative.

Paper chromatograms in the cyclohexane solvent of the ether extracts obtained during the fractionation of the urinary metabolites on alumina (table 2) showed the presence of the 3 hydroxylated metabolites of 3-FAA, as well as a trace of 3-FA by the Folin test. Elution of the corresponding spots of untreated strips into ethanol gave spectra exhibiting the maxima and minima of the appropriate compounds. The extinction at the peaks permitted the calculation of the relative amounts of the compounds in the extracts, as shown in table 2. The new 7-hydroxy derivative appears to be the major metabolite of 3-FAA, and it is excreted predominantly as the glucosiduronic acid. The 2-hydroxy compound, isolated previously as the free compound (*1*), is nevertheless present mainly as glucuronide also. It does not seem to form a sulfate. This particular compound fails to act as a chromophore and hence was not apprehended by the color tests shown in the left part of table 2. Likewise it is not included in the data of table 1. The new 2,7-dihydroxy derivative, which is formed to only a minor extent, appears as glucuronide and sulfate and also as a trace of free material. The data on sulfuric acid conjugates in table 2 were obtained by Taka-diastase hydrolysis of the fraction. Since it was shown in another instance (*9*) that this enzyme preparation hydrolyzed only about one fourth of the sulfates present, it may be necessary to multiply the data in table 2 by about 4 to obtain a realistic picture of the amounts at hand.

## DISCUSSION

The studies reported herein on certain new metabolites of 3-FAA were performed at a single dose level, a single time interval, and in a single species of animals of one sex. While it may become desirable to expand these investigations in the future and vary some of these factors, it was not deemed necessary to do so for the present. The results definitely demonstrate that rats administered 3-FAA excrete a number of mono- and dihydroxylated metabolites in certain amounts. Varying the conditions may alter the quantitative, but probably not the qualitative, aspects with single doses. The data, in any case, agree well with those of earlier reports on the metabolism of this or related compounds. Thus, in the present experiment, the total recovery of chromophoric material (34%) in the 48-hour urine of rats administered 3-FAA is in line with the 43 percent

recovered by Dyer (*11*) in 4 to 8 days in the urine of rats fed 3-FAA, especially since the greater part of a dose is excreted during the first 48-hour period.

7-OH-3-FAA is quantitatively the most important known metabolite of 3-FAA, being excreted both free and as conjugate with sulfuric and glucuronic acid. It represented about 11 percent of the dose of 3-FAA. Previously, only 2-OH-3-FAA was isolated from an ether extract of urine from rats fed 3-FAA (*1*), even though the 7-OH- and 2-OH-3-FAA exist in equal amounts as free metabolites. However, since 2-OH-3-FAA is more soluble in organic solvents than 7-OH-3-FAA, as evidenced by its higher $R_F$ value in the cyclohexane system, it would be expected to extract more readily into ether, which explains the earlier findings. It was thus demonstrated that monohydroxylation of 3-FAA took place mainly on the 2- and 7-position of the fluorene ring system. The chromatograms adduced no evidence of hydroxylation on other carbon atoms, though it could conceivably occur also on the other open *ortho* position, namely carbon 4, and also on carbon 5, in analogy to the reactions undergone by the isomeric 2-FAA. Possibly more sensitive techniques would detect such materials if present in very small amounts. In any case, biochemical hydroxylation of 3-FAA affects chiefly the positions of high-electron density contributed by the resonance phenomena in the fluorene ring system. Furthermore, the joint effect of these and the partial charges donated by the acetamido group yielded a larger amount of compound hydroxylated at the 2-(*ortho*) position than with 2-FAA where the *ortho*-positions were not part of the main fluorene ring resonance. Any metabolite hydroxylated at the methylene carbon *para* to the acetamido group, *i.e.*, at the 9-position, would not have been revealed by the methods employed in the present work. While 2-FAA has not been found to hydroxylate at that point, 9-fluorenol was a major metabolite of fluorene itself (unpublished observations of P. H. Grantham).

The dihydroxylated compound, 2,7-diOH-3-FAA, is in reality a minor metabolite of 3-FAA and is excreted largely as a sulfuric acid or glucuronic acid conjugate. Similarly, Boyland *et al.* (*13*) have isolated the sulfate conjugate of a dihydroxy derivative, 2-acetamino-6-hydroxy-5-naphthyl hydrogen sulfate, from the urine of rabbits receiving injections of 2-acetamidonaphthalene.

The observation that 7-OH-3-FAA and 2,7-diOH-3-FAA, but not 2-OH-3-FAA, are excreted as sulfates substantiates previous studies (*3, 12*) which also indicated that esterification with sulfuric acid occurred only when a hydroxy group was in the 7-position of the fluorene ring system. It is thus likely that only the 7-hydroxyl of 2,7-diOH-3-FAA was combined with sulfuric acid. However, both or only one of the hydroxy groups could have been conjugated with glucuronic acid.

The amine derived from 2,7-diOH-3-FAA is quite unstable. This fact leads to the conclusion that if deacetylation occurred during the processes of metabolism, the amine would readily be oxidized to reactive quinone-imine type compounds (*14*) or perhaps to insoluble polymers which could

appear as "protein-bound" material (*cf. 15*). However, these aspects of the problem require investigation with isotopically labeled 3-FAA.

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# Studies on the Exchange of Fluids Between Host and Tumor. II. The Blood Flow of Hepatomas and Other Tumors in Rats and Mice [1,2]

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## SUMMARY

**The blood supply of transplanted tumors was studied by two methods. The first, a direct method, is based on the cannulation of the single vein draining the blood from the neoplasm. The second, an indirect method, is based on the distribution of two indicators, $K^{42}$ and $Rb^{86}$, to the tumor. In rats, the blood supply of all tumors studied followed a uniform pattern independent of the histologic type, site of implantation, and size of the neoplasm. The results of both methods were remarkably close. The average value of the blood supply to tumors grown "tissue-isolated" was 0.14 ± 0.01 ml per hour per mg N with the direct method and 0.15 ± 0.01 ml per hour per mg N with the indirect method. The average value of blood supply to subcutaneous transplants was 0.17 ± 0.02 ml per hour per mg N. Therefore, a hepatoma had a blood supply roughly 20-fold smaller than that of the host liver. Livers regenerating after hepatectomy had the same blood supply as normal livers when the values were expressed in ml per hour per mg N. In mice, the blood supply of tumors followed the general pattern found in rats. The total amount of blood flowing through the host organ did not influence the growth rate of the transplanted tumor if [the flow reached the required low rate.—J. Nat. Cancer Inst. 27: 1465–1491, 1961.**

TO OUR knowledge the evaluation of the blood supply to a tumor is an unsolved problem. Since the growth and metabolism of the neoplastic tissue depend on its blood supply, a solution of this problem can be valuable only if the actual amount of blood flow can be determined. For this purpose, we utilized two methods which differ in technique and rationale and can be applied to various tumors grown in different species. The first, a direct method (*1*), was developed utilizing a technique previously described (*2*). The second, an indirect method, was adapted from Sapirstein's procedure (*3*, *4*). It was believed that the data would be reliable if the determinations by both methods agreed.

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[2] Presented in part at the 52d Annual Meeting of the American Association for Cancer Research, Atlantic City, N.J., April, 1961.
[3] We wish to thank Mr. J. Lieberman, Division of Research Services, National Institutes of Health, for the statistical evaluation of the data.
[4] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

## MATERIALS AND METHODS

In all experiments adult rats and mice, ranging in weight from 150 to 200 g and 25 to 32 g, respectively, were used. Table 1 summarizes the information on the tumors studied.

### Direct Method

All determinations were done under ether or sodium pentobarbital anesthesia (35 mg/kg) in heparinized rats (4 mg/kg in 0.5 ml saline) fed or fasted overnight. The peristaltic pumps used were Aminco [5] or Sigmamotor T-8.[6]

*Preparatory surgical procedure.*—At the beginning of each experiment the tumor appeared as in figure 1. A skin pouch closed with silver-nickel clips contained a paraffin envelope in which the tumor was growing. The technique for preparing such "tissue-isolated" tumors has been described (*2*). Briefly, it consists of the following steps: 1) a kidney or an ovary is "isolated" and displaced subcutaneously, leaving the vascular peduncle intact; 2) a tumor is implanted in one or both organs which are subsequently enveloped in a paraffin sack; 3) the tumor is kept growing isolated in the sack which is placed in a pouch of the subcutaneous tissue. The tumor is connected with the host only by the vascular peduncle. The procedure offers two advantages essential for our purpose: (*a*) The neoplastic mass is completely isolated from the surrounding tissues of the host (fig. 2), and (*b*) the blood supply to the tumor is carried by a single artery and vein (fig. 3).

From these tissue-isolated tumors the venous blood was collected and measured at a constant blood pressure by the experimental procedure diagrammatically presented in text-figure 1. The left carotid artery and the left and right external jugular veins were isolated through a median incision of the skin from the hyoid to the suprasternal region. A polyethylene catheter (PE50) [7] was introduced into the carotid artery and connected with a mercury manometer for blood-pressure measurement. A second polyethylene catheter (PE60) [7] was inserted into the left external jugular vein and anchored to the muscles of the clavicular region. A third catheter (PE50) [7] was introduced into the right jugular vein and guided to the tumor's vein through the superior vena cava, the right heart, and the inferior vena cava. The second and third catheters were connected by means of a peristaltic pump. All catheters had been previously filled with a solution of heparin in saline (0.1 mg/ml).

The abdominal cavity was then opened and the isolation of the peduncle of the tumor was completed. Animals with the tumor implanted in the left ovary had the left renal artery and vein tied off as near as possible to the renal pelvis, while animals with the tumor implanted in the left kidney

[5] American Instrument Co., Silver Spring, Md.
[6] Sigmamotor Inc., Middleport, N.Y.
[7] Clay Adams, 141 E. 25th St., New York, N.Y. All catheters were kept in a solution of Zephiran 1 : 2000, and washed and dried before use.

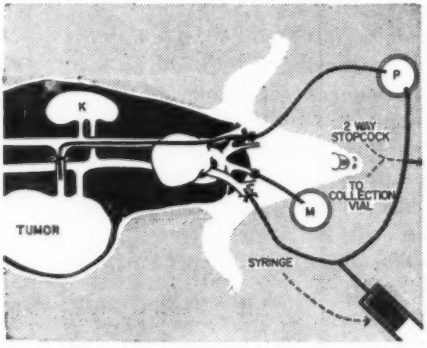
TABLE 1.—Transplanted tumors utilized for blood-flow determination

| Host | Strain | Tumor | Tumor-transplant generation (range) | Carcinogen | Reference |
|---|---|---|---|---|---|
| Rat | Buffalo/N ($F_{37}$ generation) | Hepatoma 5123 | 16–21 | *N*-2-fluorenylphthalamic acid | (*5*) |
| Rat | Buffalo/N ($F_{37}$ generation) | Fibrosarcoma 4956 | 60–82 | *N*-4-(4′-fluorobiphenyl)acetamide | (*) |
| Rat | Sprague-Dawley (random bred) | Novikoff hepatoma | 402–427 | 4-Dimethylaminoazobenzene | (*6*) |
| Rat | Sprague-Dawley (random bred) | Walker carcinoma 256 | ? | Spontaneous | (*7*) |
| Rat | Fischer/N344 ($F_{75}$ generation) | Hepatoma LC18 | 70–86 | *N*-2-fluorenylacetamide | (*8*) |
| Rat | A × C/N ($F_{65}$ generation) | Hepatoma 3683 | 73–94 | *N*-2-fluorenyldiacetamide | (*9*) |
| Rat | Osborne-Mendel ($F_{30}$ generation) | Ethionine hepatoma | 6–16 | Ethionine | (*10*) |
| Rat | (M520 × A × C)$F_1$ | Lymphosarcoma R2788 | 109–126 | Spontaneous | (*11*) |
| Mouse | C3Hf | Hepatoma P129 | 213–230 | Carbon tetrachloride | (*12*) |
| Mouse | C3Hf | Hepatoma F129 | 213–230 | Carbon tetrachloride | |
| Mouse | C3Hf | Hepatoma 134 | ? | Carbon tetrachloride | |
| Mouse | C3Hf | Osteogenic sarcoma | 54–64 | Spontaneous | (†) |
| Mouse | C3Hf ($F_{39}$ generation) | Hystiocytoma | 24–32 | Spontaneous | (†) |

*Received from Dr. H. P. Morris, National Cancer Institute.
†Received from Dr. K. C. Snell, National Cancer Institute.

had the left ovarian vessels tied off. In all rats, the left adrenal vessels connected with the renal vascular system were closed; small veins from the adipose tissue were electrocauterized. When the isolation was completed, a lace was placed around the vein of the tumor as near as possible to the inferior vena cava, and the catheter, which had been guided near the vein, was placed into it. At this point, the peristaltic pump was started and the vein of the tumor was tied off around the catheter so that all blood coming from the tumor was pumped into the left external jugular vein. The complete surgical treatment took about 30 minutes.

*Determination of blood flow.*—Ten minutes after the pump began to work, the determination of blood flow was started. By means of a switchcock the blood pumped from the tumor to the jugular vein was diverted from the circuit, collected in vials, weighed, and reintroduced into the left jugular vein. The outflow was measured continuously for

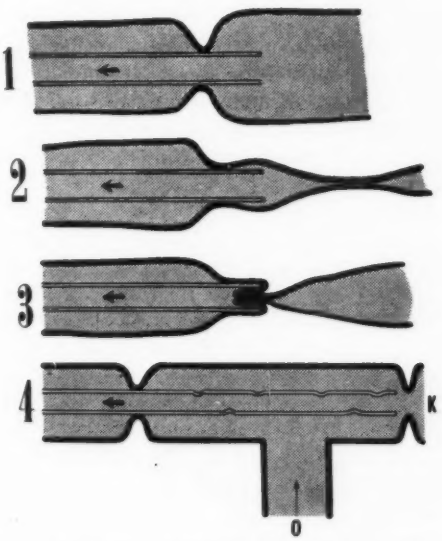



TEXT-FIGURE 1.—Diagram of the apparatus for study of blood supply of transplanted tumors by the direct method. P = peristaltic pump; M = mercury manometer. The catheter from vein of tumor to external left jugular vein has a stopcock for the collection of blood. After the blood had been weighed, a syringe was used to reintroduce it into the catheter.

30 minutes. To keep the blood pressure constant, the total amount of blood taken out of the circuit each time had to be no more than 1 ml. Therefore, when the flow was less than 30 ml per hour the blood could be collected continuously and weighed at half-minute intervals, but when the flow was larger than 30 ml per hour, the blood was collected for half a minute every 3 minutes. The syringe used for reintroducing the blood into the circuit was rinsed in saline containing heparin (1 mg/ml) which was generally sufficient to avoid clots. The amount of blood present in the catheters outside of the rat's body was about 0.5 ml. Animal and catheters were kept on an electric pad at about 37° C. At the end of each determination the pouch of the skin was opened, the isolation of the tumor checked, the peduncle dissected, and the anatomical integrity of the vessels verified.

*Testing of the experimental device.*—The crucial part of the experiment involved the relationship between the catheter and the vein of the tumor

while the pump was working. The catheter, with 2 or 3 notches at its end, was introduced into the vein for about ½ cm. At least another ½ cm of the vein was left in front of the catheter and was clearly visible under the peritoneum. The pump was started first and then the vein was tied to the catheter. When the pumping rate was too low the vein quickly enlarged, and when it was excessively high the wall of the vessel was pulled into the catheter. It was possible, however, to adjust the pump so that part of its work was used to pull the blood and part to keep the venous wall under elastic tension. When this point was reached the oscillations of the venous wall synchronized with the activity of the pump. The notches of the catheter were alternately opened and closed by the distention or collapse of the venous wall. Under these conditions the pump was actually able to remove about twice as much blood as was present in the vein, and yet operated satisfactorily when the blood flow was reduced to about one half. Only when the blood flow exceeded this limit was the vascular wall pulled into the catheter. The described conditions are shown in text-figure 2. It must be pointed out that the diameter of the renal vein was at least twice that of the catheter and therefore formed a reservoir whose blood content could be observed and used as an indicator of the pump's proper adjustment.



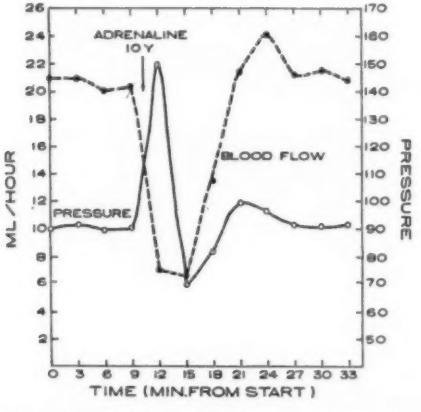

TEXT-FIGURE 2.—Behavior of venous wall when the peristaltic pump was regulated. In 1, since suction of pump is not sufficient, the vein tied to the catheter becomes turgid. In 2, suction of pump is at the right level, blood is taken away, and wall is kept under elastic tension. In 3, excessive suction of pump pulls wall of vein into catheter. In 4, proper position of catheter is shown for determinations on outflow of blood from ovarian implants. With renal vein tied off in K, catheter works in a relatively large chamber to which the blood is brought by the ovarian vein. Notches on wall of catheter are indicated only in 4.

When the pump was properly adjusted, a change in the blood flow could easily be followed as text-figure 3 shows. The blood flow of a 4.4 g Walker carcinoma 256 implanted in the kidney ranged for the first 9 minutes between 20 and 21 ml per hour with a carotid pressure of about 90 mm Hg. The intravenous injection of 10 $\mu$g of adrenalin produced an increase of pressure up to 150 mm Hg and a reduction in outflow to about 7 ml per hour. In about 6 minutes the blood pressure and the outflow returned to the initial values and in the following few minutes reached levels higher than those at the starting point.

The recorded flow reflected these changes and at the end of the experiment the pump, at the same setting, was capable of pumping 34 ml per hour from a beaker containing heparinized blood. Thus it seemed reasonable to assume that variations in the blood flow could be recorded if the range was not excessive. In fact, the blood flow from tumors was quite regular and the pump worked in a relatively narrow range. For example, when the outflow from the vein of an ovary-implanted 3.2 g Novikoff hepatoma was collected and weighed for 1 minute every 3 minutes over a period of 30 minutes, the following range of values in g was noted: 0.335, 0.328, 0.322, 0.321, 0.315, 0.319, 0.322, 0.345, 0.353, and 0.329.

The blood-flow values in tables 2 through 10 represent the average of the weights obtained in a 30-minute period. The results are expressed in ml per hour per mg N.

When the outflow of a kidney of a normal, etherized, 200 g rat was measured, the values obtained were about 30 percent lower than those procured by indirect methods in unanesthetized rats. It has been previously shown that this reduction is a result of the anesthetic (*13*); therefore the direct method, when corrected for the effect of ether, gave results comparable to the known physiological values.




TEXT-FIGURE 3.—The possibility of following changes in tumor blood flow is shown in this graph in which carotid pressure and blood flow were recorded under physiological conditions and after injection of adrenalin. *Note* simultaneous increase in pressure and decrease in blood flow.

### Indirect Method

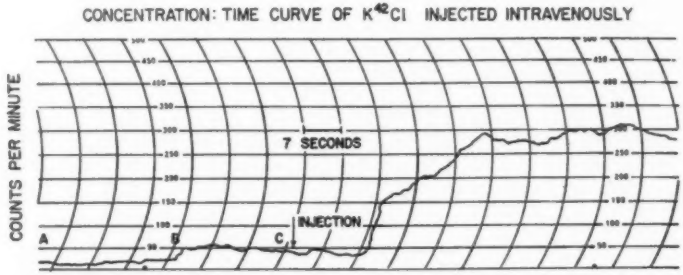
The indirect method was essentially that described by Sapirstein (*3*). It is based on the following assumption: A foreign substance, after a single, rapid, intravenous injection, will be distributed initially to the organs in proportion to their blood flow and then will be carried away by the venous drainage. For a certain period, however, the amount of substance lost by venous drainage will be negligible compared to that delivered by the artery. This period will be longest for substances which are transferred from the vascular system to the tissues with least hindrance and which have a large volume of distribution within the tissue. Sapirstein (*3*, *4*) showed that $K^{42}Cl$ is such a substance and that $Rb^{86}Cl$ could also be used. He found that after intravenous injection, the time : concentration curve of $K^{42}$ in all organs except the brain showed a steep rise during the first 10 to 15 seconds, a flattening between the 20th and 60th seconds, and variable behavior beyond this period. He interpreted the flattening of the time : concentration curve as an indication that the amount of $K^{42}$ found in each organ for about 40 seconds was approximately the amount brought by the blood flow. The fractionation of the cardiac output could be calculated when the percentage distribution of $K^{42}$ in various organs as a function of time was known and hence the blood flow of each organ could be determined.

Hydrochloric acid solutions of $K^{42}Cl$ and $Rb^{86}Cl$, obtained from Oak Ridge National Laboratory, Oak Ridge, Tennessee, were dried and redissolved in saline. The *p*H of the solution was adjusted to 7.5 with NaOH. Five to 10 $\mu$c of the radioactive material was injected as quickly as possible into the left femoral vein in 0.1 ml saline. We killed the animals by cutting through the thorax, just above the diaphragm, with a guillotine. The liver, spleen, intestine, and kidney were homogenized in a suitable amount of water, and radioactivity of each suspension was measured with a well-type scintillation counter.[8]

*Time : concentration curve of radioactive material in rat tumors.*—To apply Sapirstein's method to the determination of blood flow of the tumor, it was first necessary to determine whether the time : concentration curve in the neoplastic tissue reached a plateau as in normal organs. In a homogeneous group of normal animals one may assume that the same organ in different animals is similar enough to permit one to obtain a time : concentration curve by injecting $K^{42}Cl$ into all animals and killing them at different intervals. Unfortunately tumors are not "homogeneous" like normal organs so that the previous simple procedure could not be used. Instead, the tumor grown in a paraffin bag was taken out of the skin pouch and the paraffin envelope discarded. A lead shield was interposed between the host and the tumor with only a small opening for the vascular peduncle, a scintillation counter was placed on the tumor, and the increase in radioactivity of the tumor was determined after injection of $K^{42}Cl$ or $Rb^{86}Cl$ into the femoral vein of the host. The vascular peduncle was long enough to allow the shielding of the neoplastic mass from

[8] Analyzer model 1810 Nuclear-Chicago, Scaler model 182, and counter model DS-1.

the host (figs. 5 and 6). The increase in radioactivity of the tumor was automatically recorded. Text-figure 4 shows a typical determination. The tumor tissue reached a steady concentration at 30 to 40 seconds after the $K^{42}$ injection. Often this concentration remained constant for several minutes. Similar results were obtained in different types of tumors and also with $Rb^{86}Cl$. The diagram in text-figure 4 suggests that if a tumor-bearing animal received intravenously $K^{42}Cl$ or $Rb^{86}Cl$ and was killed after 40 seconds, the radioactivity of the tumor and of different organs is proportional to the blood flow through them. Control of the data obtained with the direct method was therefore possible with this procedure.




TEXT-FIGURE 4.—Photographic reproduction of a typical time : concentration curve obtained after intravenous injection of 10 μc $K^{42}Cl$ into a rat bearing an ovarian implant of Novikoff hepatoma. Segment A–B of the curve corresponds to the background of the room, and segment B–C corresponds to the background when the radioactive material was near the animal, immediately before injection.

It was previously shown (*2*) that tissue-isolated and subcutaneously transplanted tumors did not differ in all the biological characteristics studied. Therefore the time : concentration curve of the tissue-isolated tumors was considered valid also for the subcutaneously transplanted tumors, and the indirect method was applied to them.

*Time : concentration curve of the radioactive material in mouse tumors grown subcutaneously.*—To determine whether the indirect method could be applied to mice, the time : concentration curve for the liver, spleen,

TABLE 2.—Amounts of $Rb^{86}$ in various organs* of C3H mice at different intervals after a single, rapid, intravenous injection†

| Time (sec) | Liver | Intestine and spleen | Kidneys |
|---|---|---|---|
| 2 | 1. 3 | 7. 2 | 2. 1 |
| 5 | 3. 0 | 16. 7 | 6. 1 |
| 10 | 3. 2 | 19. 1 | 9. 9 |
| 20 | 5. 6 | 26. 6 | 8. 3 |
| 30 | 6. 3 | 22. 9 | 11. 6 |
| 60 | 5. 5 | 24. 3 | 10. 4 |

*Each value (percentage of injected dose) represents the average of 4 rats.
†Dose of 6.0 μc $Rb^{86}$ in 0.1 ml saline injected into the femoral veins.

intestine, and kidney was determined. A dose of 6 μc of $Rb^{86}Cl$ in 0.1 ml saline was rapidly injected into the femoral vein of mice and they were killed at different intervals. Table 2 shows that in the organs studied the concentration of $Rb^{86}$ reached a plateau with little variation between the 20th and 60th seconds after injection. The killing time of 40 seconds after injection was therefore chosen for the mice as it had been for the rats, and the assumption was made that the percentage of radioactive material in the tumors, as well as in different organs, corresponded to the percentage of distribution of the cardiac output.

## RESULTS

### Direct Method as Applied to Rat Tumors

#### *Effluent Blood of Ovarian Implants*

The ovary and the oviduct of adult rats were surgically isolated and the effluent blood outflow was measured without the implantation of tumors. The results obtained represent the basal values of the blood supply of the ovarian preparation and are reported in table 3. The outflow of blood was reasonably constant for different animals anesthetized with ether or pentobarbital. After each determination, the difference in weight between the right and the left ovary remained within the physiological range which was interpreted as an assurance that edema caused by disturbed circulation did not occur to an appreciable degree.

When tumors were grown in the ovarian preparation, the blood flow changed, as reported in table 4. The sizes of the tumors varied from 11 to 223 times the initial weight of the host ovary; we obtained this eval-

TABLE 3.—Blood outflow of the ovarian preparation*

| Rat No.† | Body weight | Ml per hour‡ | Ml per hour per mg N | Weight of ovary and oviduct§ (mg) | |
|---|---|---|---|---|---|
| | | | | Right | Left |
| 1 | 201 | 11. 7 ± 0. 6 | 7. 27 | 39 | 40 |
| 2 | 210 | 10. 6 ± 0. 6 | 6. 59 | 43 | 47 |
| 3 | 200 | 13. 8 ± 0. 5 | 7. 56 | 35 | 36 |
| 4 | 180 | 12. 0 ± 1. 0 | 7. 48 | 39 | 36 |
| 5 | 182 | 11. 3 ± 0. 5 | 7. 05 | 43 | 39 |
| 6 | 176 | 12. 3 ± 0. 5 | 7. 65 | 40 | 39 |
| 7 | 168 | 10. 7 ± 0. 2 | 6. 78 | 34 | 33 |
| 8 | 198 | 11. 0 ± 0. 4 | 6. 94 | 36 | 37 |
| 9 | 195 | 11. 8 ± 0. 4 | 7. 30 | 40 | 40 |

*The reported values do not correspond to the blood supply of the ovary. Our ovarian preparation consisted of the ovary, oviduct, and small amounts of adipose tissue which could not be eliminated without damage to the vascular net. Indirect measurement with $K^{42}Cl$ showed that the actual blood supply to the ovary of the adult rat is about one half the values reported in this table.

†Animals of 150 to 180 g body weight. Rats 1, 2, 3 = Sprague-Dawley strain under ether anesthesia. Rats 4, 5, 6 = A × C strain under ether anesthesia. Rats 7, 8, 9 = M520 × (A × C)$F_1$ strain under pentobarbital anesthesia (35 mg/kg).

‡± Standard error. Each number represents the average value on 30 minutes of blood-flow determination (*see* text).

§Outflow measured from organs of left side. Weights of right-side organs are given for comparison.

TABLE 4.—Blood outflow of ovarian implants

| Tumor | Rat No. | Blood flow: Ml per hr | Blood flow: Ml per hr per mg N | Right ovary + oviduct (mg wet wt) | Tumor: Wet wt (g) | Tumor: Total N (mg) | Tumor: Days from implantation |
|---|---|---|---|---|---|---|---|
| Novikoff hepatoma | 1 | 28.8 ± 0.8* | 0.53 | 31 | 2.8 | 54 | 6 |
| | 2 | 11.7 ± 0.6 | 0.15 | 31 | 4.4† | 79 | 7 |
| | 3 | 22.0 ± 0.6 | 0.24 | 42 | 5.0 | 89 | 7 |
| | 4 | 21.1 ± 0.6 | 0.22 | 39 | 5.6 | 95 | 7 |
| | 5 | 20.2 ± 0.6 | 0.22 | 60 | 6.5† | 100 | 7 |
| | 6 | 9.4 ± 0.1 | 0.08 | 40 | 6.6† | 110 | 8 |
| | 7 | 11.1 ± 0.3 | 0.07 | 39 | 8.5† | 152 | 8 |
| Hepatoma LC 18/81A | 8 | 14.3 ± 0.4 | 0.28 | 22 | 3.0† | 50 | 9 |
| | 9 | 13.5 ± 0.4 | 0.21 | 35 | 3.3 | 65 | 9 |
| | 10 | 8.2 ± 0.2 | 0.09 | 20 | 5.3† | 96 | 12 |
| | 11 | 13.0 ± 0.3 | 0.08 | 32 | 7.6† | 155 | 15 |
| | 12 | 19.2 ± 0.5 | 0.12 | 34 | 8.2† | 160 | 15 |
| Hepatoma 3683 | 13 | 10.0 ± 0.5 | 0.14 | 21 | 3.6† | 69 | 14 |
| | 14 | 14.1 ± 0.6 | 0.18 | 44 | 4.0† | 80 | 10 |
| | 15 | 17.7 ± 0.3 | 0.19 | 23 | 4.2† | 92 | 9 |
| | 16 | 5.7 ± 0.3 | 0.05 | 30 | 5.9† | 110 | 10 |
| | 17 | 14.3 ± 0.5 | 0.09 | — | 7.2 | 150 | 16 |
| | 18 | 14.7 ± 0.5 | 0.09 | 32 | 8.1† | 165 | 13 |
| Hepatoma 5123 | 19 | 12.5 ± 0.2 | 1.25 | 45 | 0.5 | 10 | 26 |
| | 20 | 9.1 ± 0.1 | 0.38 | 80 | 1.0 | 24 | 37 |
| | 21 | 16.0 ± 0.3 | 0.32 | 52 | 2.4 | 50 | 34 |
| | 22 | 20.3 ± 0.2 | 0.28 | 75 | 3.6 | 71 | 27 |
| Ethionine hepatoma | 23 | 14.2 ± 0.2 | 0.26 | 46 | 3.1 | 53 | 15 |
| | 24 | 16.1 ± 0.1 | 0.14 | 39 | 5.7† | 112 | 24 |
| | 25 | 19.4 ± 0.1 | 0.09 | 47 | 10.5† | 201 | 34 |
| Walker carcinoma 256 | 26 | 35.2 ± 1.2 | 1.40 | 39 | 1.7 | 25 | 9 |
| | 27 | 16.2 ± 0.2 | 0.70 | 47 | 1.4 | 24 | 10 |
| | 28 | 26.7 ± 1.0 | 0.89 | 38 | 1.8 | 30 | 9 |
| | 29 | 14.2 ± 0.4 | 0.23 | 58 | 2.3 | 60 | 10 |
| | 30 | 15.0 ± 0.4 | 0.27 | — | 3.3 | 56 | 13 |
| | 31 | 20.4 ± 0.8 | 0.22 | 36 | 5.1† | 94 | 14 |
| | 32 | 14.7 ± 0.4 | 0.16 | 39 | 5.2† | 90 | 15 |
| | 33 | 12.1 ± 0.3 | 0.11 | 40 | 6.2† | 110 | 14 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Lymphosarcoma R2788 | 34 | 17.8 ± 0.6 | 0.89 | 35 | 1.2 | 20 | 7 |
| | 35 | 9.8 ± 0.5 | 0.43 | 37 | 1.3 | 23 | 7 |
| | 36 | 6.8 ± 0.1 | 0.13 | 36 | 3.0 | 50 | 10 |
| | 37 | 14.4 ± 0.2 | 0.26 | 36 | 3.6 | 55 | 10 |
| | 38 | 15.0 ± 0.8 | 0.17 | 35 | 5.6† | 90 | 14 |
| | 39 | 17.0 ± 0.7 | 0.14 | 36 | 6.7† | 114 | 11 |
| Fibrosarcoma 4956 | 40 | 20.2 ± 0.5 | 1.1 | 54 | 0.8 | 18 | 13 |
| | 41 | 11.8 ± 0.3 | 0.31 | 50 | 2.0 | 38 | 14 |
| | 42 | 12.4 ± 0.1 | 0.22 | 49 | 3.2 | 56 | 14 |
| | 43 | 21.0 ± 0.7 | 0.51 | 48 | 2.6 | 41 | 21 |
| | 44 | 13.3 ± 0.7 | 0.25 | 43 | 3.0 | 54 | 25 |

*± Standard error.
†Tumor weight 100-fold or larger than that of the host ovary (*see* text).

uation by weighing the contralateral organ. In about 50 percent of the transplants, the total blood supply was higher than the basal values reported in table 3. Three significant facts arise from these data: 1) The blood supply of the host organ was often sufficient to permit the growth of a tumor about 200-fold or larger than the host organ itself; 2) the blood supply of a rapidly growing tumor like the Novikoff hepatoma was similar to the very slow-growing Hepatoma 5123; and 3) the histologic type and age of the tumor had no appreciable influence on the amount of effluent blood.

As reported previously (*2*), when the size of the ovarian implant of any tumor was 100-fold the weight of the host ovary, it was impossible to detect any residue of ovarian tissue on the histologic slides. By the assumption that in this condition the host organ had been destroyed and the blood supply measured was that required by the growing neoplasm, it is possible with the data in table 4 to group 20 determinations of tumors 100-fold the size of the contralateral ovary or even larger. The average value of blood outflow from these tumors corresponds to 0.14 $\pm$ 0.01 per hour per mg N, about 50-fold smaller than the average blood flow of the ovarian preparation.

### *Effluent Blood of Renal Implants*

The question arose whether the same trend observed in the ovarian implants was followed by tumors implanted in organs with a blood supply larger than that of the ovary. It is generally agreed and confirmed by us that the blood flow of the kidney of an adult rat is about 240 ml per hour (*13–15*), or about 20 times the flow of our ovarian preparation. The kidney was therefore chosen as the host organ and the tumors were grown tissue-isolated. In a first experiment the effluent flow of Walker carcinoma 256 was studied and the results are reported in table 5. None of the transplants had a blood outflow as large as that in the normal kidney, the flow decreased when the tumor increased in size, and a flow between 0.09 and 0.12 ml per hour per mg N was observed when the tumors weighed 12 g or more. These values were equal to the average values found in ovarian implants when the tumors were 100-fold or larger than the host ovary.

Determinations conducted on renal transplants of other types of tumors yielded the results reported in table 6. Again the blood supply decreased while the tumor was increasing in size, and, in large transplants, values between 0.1 and 0.2 ml per hour per mg N were usually observed. Table 6 shows also that the size of the tumor transplant in the kidney is not a reliable indicator of the destruction of the renal parenchyma. Hepatoma LC18, #14 of table 6, was about 8 times the size of the kidney, but the blood supply was only slightly smaller than that of a normal kidney. Figure 4 shows that the bulk of the tumor grew outside the kidney and only a small portion of the renal cortex was involved by the invasive process. The example is exceptional, but in histologic slides it is common to observe fragments of renal parenchyma well preserved in

TABLE 5.—Blood outflow of renal implants of Walker carcinoma 256

| | Blood flow | | Tumor | | |
|---|---|---|---|---|---|
| Rat No. | Ml per hour | Ml per hour per mg N* | Wet weight (g) | Total N (mg) | Days from implantation |
| 1 | 66.0 | 1.5 | 1.8 | 30 | 10 |
| 2 | 55.8 | 0.97 | 2.4 | 40 | 10 |
| 3 | 54.4 | 0.91 | 2.7 | 46 | 10 |
| 4 | 85.0 | 1.34 | 3.0 | 50 | 11 |
| 5 | 58.4 | 0.90 | 3.2 | 56 | 11 |
| 6 | 39.1 | 0.45 | 4.4 | 75 | 11 |
| 7 | 67.3 | 0.74 | 4.5 | 73 | 11 |
| 8 | 77.4 | 0.73 | 5.6 | 90 | 12 |
| 9 | 45.2 | 0.42 | 5.7 | 91 | 12 |
| 10 | 61.4 | 0.54 | 6.1 | 102 | 12 |
| 11 | 73.0 | 0.61 | 6.4 | 107 | 12 |
| 12 | 41.6 | 0.34 | 6.6 | 108 | 12 |
| 13 | 62.7 | 0.48 | 7.3 | 120 | 12 |
| 14 | 38.8 | 0.24 | 8.7 | 145 | 13 |
| 15 | 38.1 | 0.21 | 9.5 | 160 | 13 |
| 16 | 22.6 | 0.12 | 10.2 | 162 | 13 |
| 17 | 27.8 | 0.13 | 11.3 | 184 | 13 |
| 18 | 69.5 | 0.33 | 11.7 | 186 | 13 |
| 19 | 25.0 | 0.11 | 12.0 | 202 | 14 |
| 20 | 27.1 | 0.12 | 12.3 | 210 | 14 |
| 21 | 32.0 | 0.12 | 14.2 | 270 | 14 |
| 22 | 27.2 | 0.10 | 14.4 | 275 | 14 |
| 23 | 23.9 | 0.09 | 14.6 | 270 | 15 |
| 24 | 41.5 | 0.12 | 18.2 | 310 | 15 |

*The blood flow of a normal kidney is about 10.0 ml per hour per mg N.

large, old tumors which are not very invasive. Tables 5 and 6 demonstrate, therefore, that a correct evaluation of the blood flow of the tumor requires a system in which any residue of the host's supply can be excluded. For this reason, tissue-isolated ovarian implants larger than 100-fold the weight of the host ovary appear to be the best preparations.

## Indirect Method as Applied to Rats and Mice

### *Indirect Method of Blood-Flow Determination in Rats Bearing Tissue-Isolated and Subcutaneously Implanted Tumors*

Four groups of rats were used in the first experiment. About 8 hours after the last feeding, all animals received an intravenous injection of 10 $\mu$c of $K^{42}Cl$ in 0.1 ml saline and were killed 40 seconds later. Group 1, composed of 12 normal rats, was used as the control. Group 2 consisted of 6 rats hepatectomized about 60 hours before injection (70% removal). Group 3 included 15 adult animals bearing ovarian transplants of the following tumors: Hepatoma LC18, Hepatoma 3683, Novikoff hepatoma, Lymphosarcoma R2788, and Walker carcinoma 256. Each tumor was transplanted into 3 rats; the size of the tumors varied between 3.0 and 9.2 g. Group 4 comprised 21 rats bearing usual subcutaneous transplants. Table 7 gives a summary of the determinations. There was no significant difference between the blood outflow of isolated ovarian transplants and the outflow of subcutaneous transplants. The

TABLE 6.—Blood outflow of renal implants

| Tumor | Rat No. | Blood flow: Ml per hour | Blood flow: Ml per hour per mg N | Right kidney wet wt (g) | Tumor: Wet wt (g) | Tumor: Total N (mg) | Tumor: Days from implantation |
|---|---|---|---|---|---|---|---|
| Novikoff hepatoma | 1 | 135 ± 2.0* | 5.4 | 0.71 | 1.7 | 25 | 6 |
| | 2 | 150 ± 3.0 | 3.0 | 0.81 | 2.9 | 50 | 6 |
| | 3 | 115 ± 2.0 | 2.5 | 0.72 | 2.3 | 46 | 7 |
| | 4 | 74 ± 1.5 | 1.2 | 0.62 | 3.4 | 60 | 8 |
| | 5 | 62 ± 3.0 | 0.87 | 0.60 | 4.2 | 71 | 8 |
| | 6 | 67 ± 1.3 | 0.80 | 0.67 | 5.3 | 84 | 9 |
| | 7 | 14 ± 0.4 | 0.13 | 0.60 | 6.6 | 105 | 9 |
| | 8 | 22 ± 0.6 | 0.15 | 0.71 | 8.3 | 142 | 10 |
| | 9 | 35 ± 0.9 | 0.20 | 0.75 | 9.6 | 170 | 10 |
| | 10 | 11 ± 1.0 | 0.05 | 0.65 | 12.3 | 206 | 11 |
| Hepatoma LC 18/81A | 11 | 61 ± 3.0 | 1.0 | 0.60 | 3.3 | 61 | 11 |
| | 12 | 38 ± 1.4 | 0.70 | 0.65 | 3.3 | 56 | 11 |
| | 13 | 53 ± 3.0 | 0.61 | 0.70 | 5.6 | 86 | 11 |
| | 14 | 206 ± 4.0 | 2.06 | 0.81 | 6.3 | 100 | 11 |
| | 15 | 43 ± 2.0 | 0.36 | 0.72 | 7.3 | 120 | 13 |
| | 16 | 34 ± 2.0 | 0.20 | 0.80 | 10.1 | 172 | 15 |
| | 17 | 25 ± 1.5 | 0.12 | 0.73 | 13.4 | 214 | 17 |
| Hepatoma 3683 | 18 | 58 ± 2.0 | 2.63 | 0.65 | 1.6 | 22 | 7 |
| | 19 | 68 ± 3.0 | 2.83 | 0.72 | 1.7 | 24 | 7 |
| | 20 | 53 ± 2.0 | 1.76 | 0.68 | 1.8 | 30 | 7 |
| | 21 | 44 ± 0.5 | 1.51 | 0.75 | 2.0 | 29 | 8 |
| | 22 | 84 ± 3.0 | 2.80 | 0.84 | 2.1 | 30 | 8 |
| | 23 | 67 ± 2.0 | 1.67 | — | 2.7 | 40 | 9 |
| | 24 | 24 ± 1.0 | 0.23 | 0.90 | 6.3 | 105 | 12 |
| | 25 | 18 ± 1.0 | 0.11 | 0.95 | 9.2 | 161 | 13 |

*± Standard error.

TABLE 7.—Blood-flow determinations with the indirect method in rats bearing ovarian tissue-isolated and subcutaneous transplants of various tumors*

| | Tumors | Liver | Kidney |
|---|---|---|---|
| Group 1 | | | |
| Control | — | 3.3 ± 0.23 | 6.9 ± 0.27 |
| Group 2 | | | |
| Hepatectomy | — | 3.5 ± 0.23 | 7.3 ± 0.31 |
| Group 3 | | | |
| Ovarian implants | 0.15 ± 0.01 | 3.2 ± 0.14 | 5.9 ± 0.27 |
| Group 4 | | | |
| Subcutaneous implants | 0.17 ± 0.02† | 3.0 ± 0.12 | 5.9 ± 0.24 |

*Numbers represent ml per hour per mg N (± standard error). We calculated them by assuming a physiological cardiac output of 205 ml per minute (*see* text).
†Average value from the determinations of table 8.

values obtained for the blood outflow of both types of transplants were 0.15 ± 0.01 and 0.17 ± 0.02 ml per hour per mg N, which were surprisingly close to the average value of 0.14 ± 0.01 ml per hour per mg N obtained with the direct method in tissue-isolated ovarian transplants. No statistically significant difference among the three values was found.

The average amount of blood flowing in the livers of tumor-bearing rats did not differ significantly from that in the normal animals. Regenerating livers had the same amount of blood supply when expressed as per mg of tissue nitrogen and an over-all reduction when expressed as amount of blood per total organ.

In a second experiment, the blood flow of subcutaneously transplanted tumors was studied. With the indirect method it was possible to utilize tumors larger than those studied with the direct method because there was no need to keep the neoplasms isolated in paraffin envelopes. Table 8 shows that in subcutaneous transplants also the blood flow usually remained around 0.1 to 0.2 ml per hour per mg N. Again it can be seen that the histologic type and the growth rate of the neoplasm is not a factor in the pattern of the blood supply. Furthermore, the indirect method confirms the rather uniform blood outflow which was observed with the direct method.

It should be re-emphasized that a reliable determination of the blood flow in tumors can be obtained only if normal tissues are excluded from the neoplasm. In our experience all intramuscular transplants are unsuitable because large fragments of muscle can survive in old tumors.

### *Indirect Method of Blood-Flow Determinations in Mice Bearing Subcutaneously Transplanted Tumors*

Twenty-five mice bearing Hepatoma 129 (carried in two lines, P and F), Hepatoma 134, osteogenic sarcoma, or a hystiocytoma were given intravenous injections of 6.0 μc $Rb^{86}Cl$ in 0.1 ml saline. The amounts of $Rb^{86}$ found in the tumors, in the livers plus the organs of the portal system, and in the kidneys are reported in table 9. These amounts are expressed as percentages of the injected dose per g of wet tissue. The hepatomas contained an amount of indicator roughly 15-fold smaller than that in the livers and 30-fold smaller than that in the kidneys. The average weight

TABLE 8.—Blood flow of subcutaneously transplanted tumors from $K^{42}$ distribution*

| Rat | | Tumor | | |
|---|---|---|---|---|
| Number | Body weight + tumor (g) | Type | Weight (g) | Ml per hour per mg N† |
| 1 | 189 | Hepatoma 3683 | 2.7 | 0.04 |
| 2 | 185 | " | 3.8 | 0.17 |
| 3 | 183 | " | 4.4 | 0.09 |
| 4 | 174 | " | 6.9 | 0.09 |
| 5 | 252 | " | 35.0 | 0.18 |
| 6 | 233 | " | 35.4 | 0.14 |
| 7 | 250 | " | 39.3 | 0.15 |
| 8 | 248 | " | 53.5 | 0.13 |
| 9 | 276 | " | 70.0 | 0.22 |
| 10 | 160 | Hepatoma LC18 | 2.0 | 0.13 |
| 11 | 150 | " | 10.2 | 0.15 |
| 12 | 150 | " | 10.8 | 0.16 |
| 13 | 176 | " | 19.2 | 0.27 |
| 14 | 159 | Novikoff hepatoma | 2.3 | 0.20 |
| 15 | 164 | " | 4.4 | 0.09 |
| 16 | 152 | " | 21.8 | 0.4 |
| 17 | 143 | Lymphosarcoma R2788 | 13.1 | 0.04 |
| 18 | 148 | " | 14.4 | 0.19 |
| 19 | 190 | Walker carcinoma 256 | 37.3 | 0.22 |
| 20 | 185 | " | 51.7 | 0.19 |
| 21 | 207 | " | 67.4 | 0.25 |

* Single, rapid, intravenous injection of 10 $\mu$c of $K^{42}$ $\mu$c in 0.1 ml saline. Animals killed 40 seconds after injection.
† Calculated by assuming a physiological cardiac output of 205 ml per minute.

of the hepatomas was 2.5 g in a range of from 0.3 to 5.2 g. The distribution of the indicator to the osteogenic sarcomas was in the same order of magnitude (compared with the livers) for tumors of an average weight of 2.8 g in a range of from 0.5 to 3.5 g. The histiocytomas received a larger supply. The average weight of the tumors was 1.5 g in a range of from 0.2 to 2.5 g. As observed for the rats, the tumor-bearing mice had a fractional distribution of blood to the liver in a physiological range. In two groups of animals, anemia probably caused the lower than normal blood supply to the kidneys (*16*, *17*).

TABLE 9.—Amounts of $Rb^{86}$ recovered after intravenous injection in mice*

| | Tumor | Liver | Kidneys |
|---|---|---|---|
| Hepatoma 129P | 1.1 | 18.1 | 34.5 |
| Hepatoma 129F | 1.2 | 17.3 | 36.4 |
| Hepatoma 134 | 1.4 | 19.2 | 35.5 |
| Osteogenic sarcoma | 0.8 | 15.0 | 22.9 |
| Histiocytoma | 2.5 | 18.0 | 16.9 |

* Each value (percentage of the injected dose per g of wet tissue) represents the average of 5 mice. All mice were killed 40 seconds after receiving $Rb^{86}Cl$ injection.

To our knowledge, the cardiac output of the mouse has not been determined, and therefore the distribution of the indicator cannot be calculated, as for rats, in terms of ml per hour per mg N. However, our experiments showed that the neoplastic tissue in the mice also had a low blood supply which appears to be nearly independent of the type of tumor.

### Relationship Between Blood Supply and Tumor Growth

The data presented in the first two sections suggest that there is a characteristic slow blood flow in tumors which remains relatively constant during the period of tumor growth and is independent of the flow rate of the vascular bed into which the tumors are implanted. If this is true, the blood supply of the host organ should not influence the growth rate of a transplanted tumor. To test this hypothesis, the following experiment was performed: Ten-mg fragments of the same tumor were implanted into the isolated right ovary and left kidney of the same rat and the tumors were grown tissue-isolated. The blood supply of the ovarian implant was 12 ml per hour while that of the kidney implant was 240 ml per hour. After a suitable period, depending on the growth rate, both tumors were removed simultaneously and the amount of neoplastic tissue was evaluated. Thus, in the same animal, the same amount of tumor was implanted into two organs having a 20-fold difference in blood supply. Both tumors were grown in a sack of paraffin isolated from the surrounding organs and from neighboring vascular systems. Table 10 shows that the amount of neoplastic tissue grown in the ovary was equal to, or larger than, the amount grown in the kidney. The over-all picture indicates that the amount of blood flowing through the host organ does not influence the growth rate of the tumors studied if the flow reaches the required low rate.

TABLE 10.—Amounts of neoplastic tissue grown in organs having different levels of total blood supply

| Tumor* | Days from implantation | Total amount of neoplastic tissue † | | | |
|---|---|---|---|---|---|
| | | Renal implants | | Ovarian implants | |
| | | Wet weight (g) | Total N (mg) | Wet weight (g) | Total N (mg) |
| Novikoff hepatoma | 11 | 37. 7 | 754 | 77. 0 | 1511 |
| Hepatoma 3683 | 12 | 37. 9 | 765 | 45. 9 | 916 |
| Hepatoma LC18 | 12 | 22. 7 | 454 | 31. 4 | 630 |
| Hepatoma 5123 | 44 | 37. 0 | 743 | 27. 0 | 540 |
| Walker carcinoma 256 | 13 | 49. 3 | 988 | 50. 2 | 996 |
| Lymphosarcoma R2788 | 13 | 48. 8 | 961 | 43. 4 | 890 |

*Ten animals in each group.

†We evaluated the amount of neoplastic tissue by subtracting from the wet weight or the total nitrogen of the tumor the wet weight or the total nitrogen of the contralateral kidney or ovary.

## DISCUSSION

The blood supply to tumors has been the subject of many investigations, but most of the work has been devoted to the morphological aspect of the problem (*18–22*). Furthermore, the blood supply and the vascularity of the tumors have often been confused, and the interpretations of data have therefore been misleading.

An indirect evaluation of the blood flow was attempted by the comparison of the oxygen content of the venous and arterial blood that serves areas with and without neoplasms (*23*), by the evaluation of the effect on the tumor of a reduction in peripheral blood pressure (*24*), distribution of Lissamine green (*25*, *26*), and by the determination of the oxygen tension in the neoplastic tissue (*27*). None of these methods permitted a determination of the actual amount of blood flowing through a growing neoplasm; to our knowledge, the data reported in this paper are the first in the literature.

Under our experimental conditions, ovarian transplants in the rat offered the best opportunity to work with a tumor in which the residues of the host organ were absent when the tumor was larger than 4.0 g (*2*). It can be assumed that the blood flow measured in such a tumor corresponded to the "actual" blood flow of the neoplastic tissue. The average value with the direct method was 0.14 ± 0.01 ml per hour per mg N and with the indirect method, 0.15 ± 0.01 ml per hour per mg N. These remarkably close results were confirmed by the renal transplants. In them, the blood flow was very high when the tumors were small, but leveled off around 0.1 to 0.2 ml per hour per mg N when the tumors were large. It seems reasonable that in small tumors the measured blood flow was the supply to the tumor and kidney. When the kidney was destroyed by the growing neoplasm, the blood flow closely accounted for the actual supply to the neoplastic tissue and at this point renal and ovarian implants had the same blood supply.

Only the indirect method could be used on the subcutaneously transplanted tumors, and in them the average blood flow measured was 0.17 ± 0.02 ml per hour per mg N. In view of the large difference in size of the tumors studied, this result was remarkably close to the data obtained in the tissue-isolated preparations.

Besides the small blood supply, the neoplastic tissue had another unexpected characteristic, *i.e.*, the tendency toward uniformity in the blood flow, regardless of histologic type, size of the neoplastic mass, and the organ in which the tumor grew. The obvious question at this point is: What regulates the new formation of tumor vessels and the blood flow in them? Some data on this problem will be reported in a following paper of this series.

It is commonly believed that the blood supply of a tumor is high. The facts in support of such an opinion come from many sources: In histologic slides many tumors appear to be rich in vessels, a large vascular plexus has often been described around subcutaneous tumors, and it seems obvious that a rapidly growing tumor needs a large supply of "nutrients," therefore, a large blood supply. Actually, no one knows the minimum requirement of blood for growing tissues. Table 7 shows that a liver reduced to about 40 percent of its mass and actively regenerating has a blood supply not significantly higher than a normal liver when evaluated per mg N and lower than normal if compared with the over-all supply of the entire organ. Certainly the increased rate of cell proliferation in the

liver does not require a proportional increase in blood supply. The normal blood flow of the liver is sufficient to insure the supply needed for the regenerating process, which is indirect evidence that the fraction of blood supply utilized by the liver for the regenerating process is relatively small.

All our work was done on transplanted tumors; we have no data to demonstrate that the results can be applied to primary tumors. Our data, however, show that many types of neoplastic tissues have a relatively uniform blood supply which does not change through many transplant generations. During this investigation, 25 Novikoff hepatoma transplant generations were prepared and the blood flow remained in the same range for the first and the last transplants. Since it seems certain that the blood supply of neoplastic tissue does not change in subsequent transplantations, it is probable that primary and transplanted tumors have the same blood supply.

A low supply of blood and the tendency toward a uniform rate of blood flow in different histologic types of tumors was observed in mice also. The amount of information we have does not allow an exact evaluation in terms of ml per hour per mg N such as was made for the rat, but the comparison between the blood supply of normal liver and hepatomas showed a 15-fold reduction in the tumors.

If the blood flow of hepatomas in rats and mice follows the same laws which regulate the general circulation, it can be expected that a substance injected into the host will circulate roughly 20 times through the liver before passing once through the hepatoma. In view of these facts, the effectiveness of certain chemotherapeutic agents may have to be re-evaluated because the dose received by the tumor per unit of time may be very small. In addition, some metabolic findings that have been explained as properties of the tumor cell may, in part, be secondary to the conditions of exchange of fluids between host and tumor.

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# PLATES

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PLATE 212

FIGURE 1.—Skin pouch closed with silver-nickel clips, which contains tumor enveloped in paraffin sack. The blood-flow determination was performed with tumor in this condition.

FIGURE 2.—Paraffin sack which envelopes tumor transplanted in an ovary. *Note* complete isolation of tumor and vascular peduncle which connects neoplasm with host.

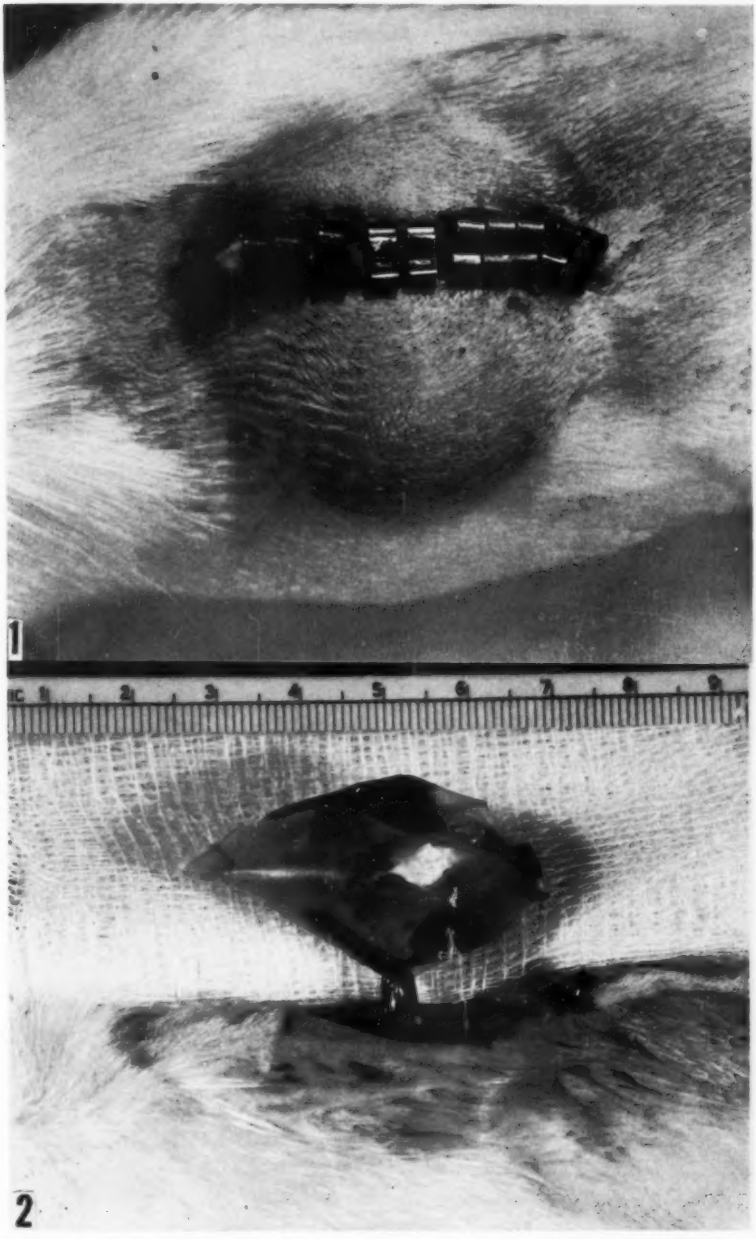
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PLATE 213

FIGURE 3.—Kidney-transplanted 5.2 g Walker carcinoma 256 grown in a paraffin sack. Paraffin envelope was discarded, tumor was placed on a filter paper, and renal artery and vein were dissected to show that the blood supply of preparation was dependent on two vessels only. *Note* smooth surface of tumor which does not adhere to surrounding tissues because of protection afforded by paraffin envelope.

FIGURE 4.—A 6.3. g Hepatoma LC18 implanted in kidney. *Note* that tumor grew on surface of the kidney from which it received its supply of blood. Histologically only a very thin layer of renal cortex was involved and there was no sign of invasion along vessels. External surface of tumor was covered by a paraffin sack (*see* text).

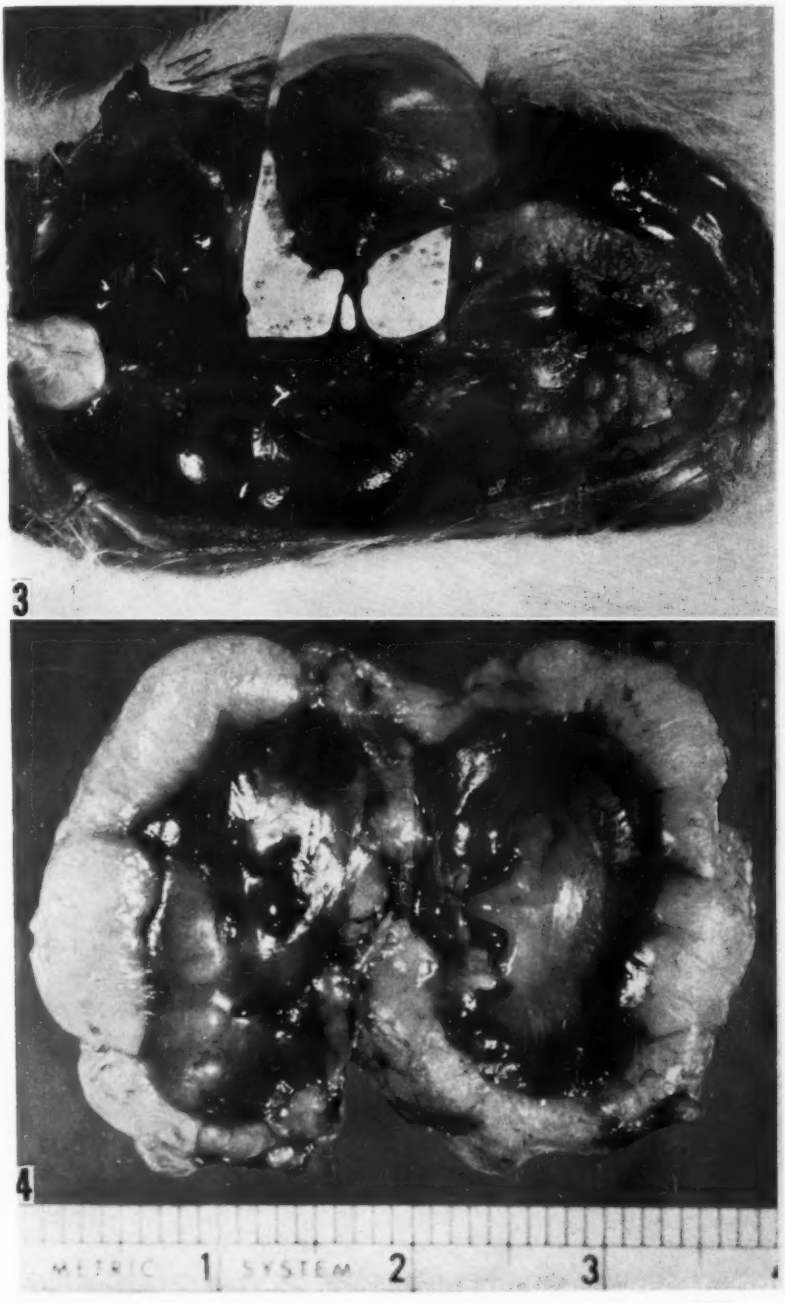
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PLATE 214

FIGURE 5.—Ovarian implant of a 3.6 g Novikoff hepatoma displayed under a scintillation counter. *Note* vascular peduncle connecting tumor to vascular system of host.

FIGURE 6.—Complete setup for time: concentration curve determination after intravenous injection of $K^{42}$ or $Rb^{86}$. *Note* that length of vascular peduncle allowed interposition of a lead shield.

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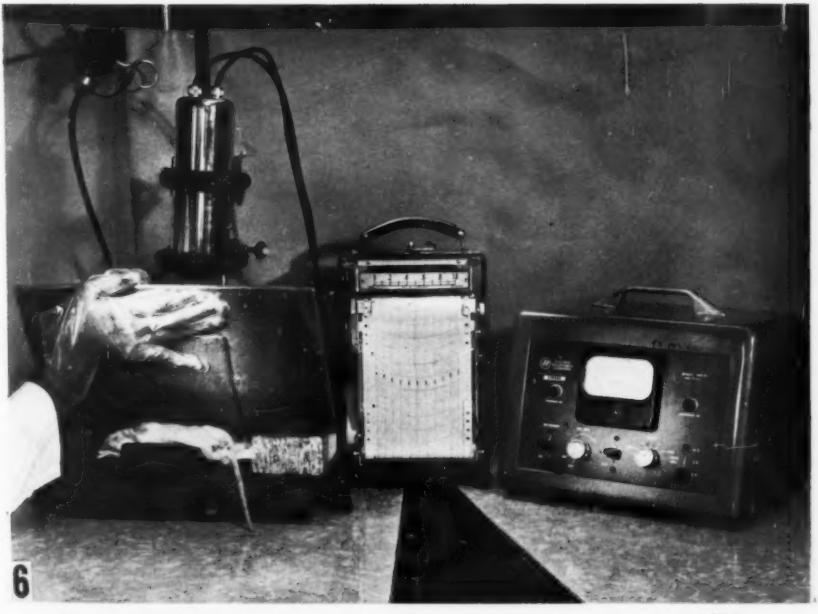
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# Hormone-Induced Tumors. II. Flank-Organ Epithelioma of the Syrian Hamster *In Vitro* [1, 2]

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SUMMARY

**In dispersed cell culture, the androgen-estrogen-induced, dependent epithelioma of the flank organ of the Syrian hamster behaves similarly in medium with or without exogenous hormones. In organotypic culture, significant growth, *i.e.*, mitosis, occurs only in hormone-enriched medium. Survival of tumor tissue within the 1-month culture period is significantly better in the hormone-treated organ cultures, though appreciable quantities of healthy-looking tissue are occasionally present in those receiving no treatment. This pattern of response in organ culture parallels the pattern observable *in vivo*: When exogenous androgen-estrogen is removed, tumor growth ceases, but subsequent decrease in size is extremely gradual and slight, even over a period of 300 to 400 days. This response of the flank-organ tumor in organ culture is essentially similar to that of the previously described estrogen-induced dependent carcinoma of the hamster kidney, which failed to grow or survive beyond a limited time in hormone-free medium. The behavior of these tumors *in vivo* seems to be paralleled to a certain extent in organotypic culture.—J. Nat. Cancer Inst. 27: 1493–1502, 1961.**

THE ANDROGEN-ESTROGEN-induced, dependent epithelioma of the flank organ [4] of the Syrian hamster, *Mesocricetus auratus*, originally described by Kirkman (*1*), was subsequently investigated in tissue culture (*2*), with methods successfully employed by Murray (*3*) and Murray and Stout (*4*) in the classification of certain human tumors of unknown origin. Because of the stringent growth requirement of this tumor for the inducing hormones *in vivo* (*1*), estrogens and androgens were added to the tissue-culture medium in an attempt to promote maximal cellular outgrowth. It was determined that estrogen alone had no stimulating effect on cell cultures of the flank-organ tumor; however, attempts to

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[3] The assistance of Miss Sumiko Sakaguchi and Mrs. Alice Dodge is gratefully acknowledged.

[4] Hamster flank organs are small, slightly raised, heavily pigmented scent glands. Although present at birth, they do not attain full size until sexual maturity. They are androgen-sensitive and most conspicuous in the sexually mature male. Histologically, they consist of many hypertrophied sebaceous glands and hair follicles interspersed with many melanocytes. For a more detailed account, *see* descriptions of Lipkov (*5*) and Lochbrunner (*7*).

test the effects of testosterone, either alone or in combination with estrogen, were frustrated by the fact that the androgen available was highly toxic.

A more thorough study (*2*) of an estrogen-induced, dependent carcinoma of the hamster kidney (*5*) revealed that while cell cultures flourished in the absence of estrogen, organ cultures, where tissue relationships are preserved, must be hormone-treated to grow and/or survive. It has since been possible to obtain sufficiently nontoxic testosterone to permit a further evaluation of the hamster flank-organ tumor, the results of which are presented in this paper.

## MATERIALS AND METHODS

Primary flank-organ tumors, serial subpannicular transplants, and lung metastases served as sources of culture tissue. In all cases, transplants and metastases were taken only from tumor lines that had been routinely checked for the possible acquisition of autonomy.

The organ-culture method used was the standard "watchglass rayon-acetate raft" technique (*8*) modified by the addition of a bed of 3 mm glass beads to support the raft (*2*). Cultures were incubated at 37° C in a humidified 5 percent $CO_2$ atmosphere at a *p*H of 7.4 ± 0.1. Cell cultures were established in Maximov slides, roller tubes, and static tubes, with the use of cut fragments or disaggregated tissue obtained by being minced and digested with collagenase or trypsin (*2*) according to the method of Lasfargues (*9*).

Morgan's medium 199 supplemented with 10 percent freshly extracted 11-day chick embryos ($EE_{50}$) was the principal medium used, though similar results were obtained in trials with Healy's medium 1066 and Eagle's basal medium. Two hundred units of sodium penicillin G, 50 μg of streptomycin sulphate, and 0.015 mg of phenol red per ml of medium were routinely used. We purchased diethylstilbestrol and testosterone from the Nutritional Biochemicals Corporation, Cleveland, Ohio, and introduced them into the medium as microcrystalline suspensions, prepared by dissolving the hormones in ethanol and injecting them directly into balanced saline solution (BSS). A stock suspension containing 1 mg of each hormone per ml was diluted to the desired concentrations in medium 199 plus 10 percent $EE_{50}$ immediately before explantation or subsequent feedings. Alcohol concentration was held to approximately 0.2 percent in all cultures.

A standard organ-culture experiment consisted of four series of 4 watchglass cultures, each containing 6 randomly selected tumor fragments of approximately 2 $mm^3$. Five to 10 explants were fixed immediately for histological appraisal of the tumor prior to explantation. The control series (4 dishes—24 explants) was given no hormones; the remaining 3 were treated with medium containing 5, 10, or 25 μg of both diethylstilbestrol and testosterone. Medium was changed twice weekly

after routine inspection for contamination, abnormal *p*H values, and general appearance of the tissue fragments. One fragment of tissue was removed from each culture at 3, 7, 9, 16, 23, and 30 days. Each collective sample was fixed in Bouin's solution and processed simultaneously for histological examination according to methods described previously (*10*). Staining was done with Harris' hematoxylin and eosin unless otherwise specified.

Samples, serially sectioned at 7 $\mu$, were examined to determine relative amounts and distribution of surviving tissue, cellular pathology, *e.g.*, vacuolization, pyknosis, chromatin clumping, loss of basophilia, abnormal nucleoli, abnormal division figures, multinuclear cells, and nuclear and cellular pleomorphism. The total number of mitotic figures per explant was counted. To avoid counting division figures twice, alternate sections were examined; only sharply distinguishable mitoses were recorded.

The cell cultures were also divided into four groups, one receiving no hormone treatment, the remaining receiving 5, 10, or 25 $\mu$g of the hormone mixture per ml. Cultures were examined daily, fed twice a week, and when preserved, were fixed in absolute methanol and stained with Jenner-Giemsa.

## RESULTS

### Organ Cultures

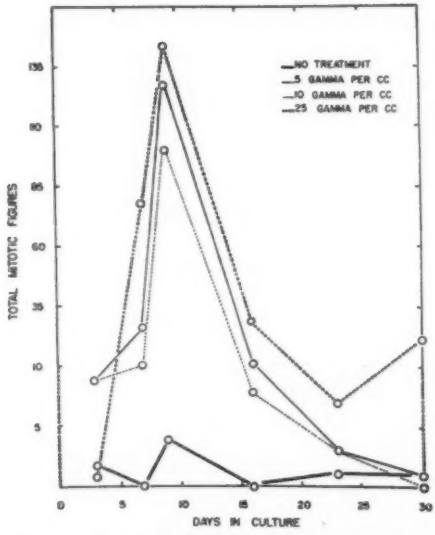
The results reported here were derived from studies made on 12 organ-culture experiments conducted, as donor material became available, over more than 2 years. Technical failures, the selection of less than optimal tumor tissue,[5] etc., resulted in the premature termination of half of the experiments, although useful data concerning behavior during the first 10 days were salvaged in 3 of these. Of those which were completed, the 1 which most clearly characterized the results was selected as the basis of the following description.

The cut surfaces of flank-organ tumor explants repaired within 72 hours, and, usually, the original cubes of tissue became essentially spherical or ovoid. Within 72 hours in culture, with rare exceptions, the cells in the central mass of the explant died, leaving a dead connective-tissue skeleton supporting a cortical layer of living tissue. This cortex averaged approximately 165 $\mu$ in thickness; however, it was typically thinner adjacent to the raft surface, about 50 $\mu$. Thus, the average thickness of the remaining two thirds or so of the cortex was proportionately greater, averaging 280 $\mu$. Seldom was the distribution of cortical tissue symmetrical: In the majority of explants there was one or more areas of surviving tissue considerably more extensive than the remainder. In a few instances, clusters or nodules of perfectly healthy-looking tumor

[5] The mitotic activity observable in any given hormone-treated organ culture is definitely related to the mitotic activity in the donor tumor. If no or few mitoses are found in "initial" samples, no or few mitoses are subsequently found in organ cultures, even though survival may be excellent.

tissue were found scattered throughout the interior of an explant. Whether this condition was related to some difference in the tumor-cell population or to other factors, *e.g.*, medium diffusion through vascular channels, has not been determined.

The quantities of living tissue present in explants after 72 hours of culture, with or without hormone treatment, were similar, as was the appearance of the tumor cell—degree of basophilia, diameter of nuclei, and number of nucleoli. When compared to control samples taken before explantation, the number of mitoses in the tissue after 72 hours was diminished.[6] Although the mitotic counts were low (text-fig. 1), more dividing cells were found in cultures receiving 5 and 10 $\mu$g per ml doses of hormone mixture than in those that were untreated or which received the heaviest dose (25 $\mu$g per ml).




TEXT-FIGURE 1.—Comparison of mitotic activity observed in untreated and androgen-estrogen-treated organ cultures of flank-organ epithelioma. (*Note* that lower part of graph is expanded.)

After 72 hours in culture, the amounts and morphological characteristics of surviving tissue were similar. At each hormone level mitosis fell to a very low value, but more figures were found in fragments treated with intermediate levels of hormones than in those receiving no treatment or relatively heavy treatment. In view of the over-all reduction in mitosis after 72 hours, the differences observed may be of questionable significance.

After 7 days in culture, the cortical zones of all explants were reduced in average thickness, the upper two thirds averaging 220 $\mu$. The irregular

[6] Although a mitotic index for tumor tissue *in vivo* was not computed, mere inspection readily reveals that mitotic activity *in vivo* far exceeds that observed in cultured tumor tissue at the end of the 3d day.

distribution of tissue noted at 72 hours was more pronounced; areas of normal-looking tissue interspersed in thinner, less healthy cortex in which chromatin clumping, pyknosis, and cytoplasmic vacuolization were frequently noted. Although the amount of viable tissue in treated and untreated fragments was essentially the same, cells in the untreated tissue tended to be about 1 $\mu$ smaller in diameter. Mitosis was limited to treated cultures in which it occurred principally in the healthier areas. Occasionally figures were found in areas containing scanty amounts of tissue. Division frequency in untreated cultures approached zero, and, indeed, in the experiment being described, not a single dividing cell was seen. Frequency of division in hormone-treated cultures varied; the largest number occurred in those treated with the 25 $\mu$g per ml mixture (text-fig. 1).[7]

A slight general decline in the number of cells took place during the second 72 hours of culture. While amounts of tissue were essentially similar in treated and untreated cultures, cell division occurred far more frequently in cultures receiving hormone treatment.

Fragments sampled on the 9th day of culture showed more pronounced differences between treated and untreated cultures than at any other time during the experiment. Untreated fragments, although they contained substantial quantities of tumor tissue, were by no means as vigorous as those which had been hormone-treated. Cortices were generally thinner in those receiving no hormone treatment; at least a part of this thinning may be accounted for by the fact that the average cell diameter was approximately 3 $\mu$ less than in cultures receiving hormone treatment; pyknosis and vacuolization were also more common. Even so, the occurrence of scattered nodules and strands of quite normal-appearing cells was observed; however, cell division was rare, only 4 division figures being present.

Uneven distribution of "active" nodules was more apparent in treated fragments, and it was in these that the mitotic activity reached its peak (text-fig. 1), the maximum numbers occurring in fragments treated with 25 $\mu$g per ml doses.

All fragments at the 9th day had a cortex of viable tissue; untreated fragments had less cortex than those receiving treatment. Mitosis was negligible in untreated cultures, but reached its peak frequency in cultures receiving the maximal hormone dose.

By the 16th day, the amounts of viable tumor tissue were approximately the same as described for the samples taken on the 9th day. Untreated cultures appeared slightly "inferior" to any of those treated with hormones, though there were areas which compared favorably with fresh, uncultured samples. No mitoses were found in untreated cultures; the largest number was found in fragments treated with the heaviest dose of hormones (text-fig. 1).

After 23 days, the amount of viable tissue was slightly less in the

[7]Attempts to elicit even stronger responses by increasing the hormone level were unsuccessful; 50 $\mu$g per ml of the hormone mixture caused severe damage.

untreated fragments and was greatest in those under 25 μg per ml hormone treatment. Mitotic activity was generally quite low, more figures occurring in the treated tissue (text-fig. 1).

On the 30th day, untreated samples showed appreciable quantities of surviving tissue; however, the treated explants contained more, a maximal amount being found in tissue receiving the heaviest hormone dose. Significant numbers of mitoses were found only in the tissue under the heaviest treatment (text-fig. 1).

By the 9th day or so, the peak of survival and growth had obviously been passed. There was a gradual decline during the remainder of the culture period. Surviving cells were found in all tissue on the 30th day of culture. Mitosis was limited almost exclusively to the tissue under the heaviest hormone treatment.

### Cell Cultures

As previously reported (*2*), flank-organ tumor tissue planted in or on the surface of a plasma clot produced a coronal outgrowth of cells within 48 to 60 hours. The pattern of outgrowth was more epithelioid when enzyme-dissociated tissue was "seeded" on the surface of an extremely thin clot. Estrogen, androgen, or both hormones in combination had no apparent effect on the rate of immigration, number of cell divisions, individual cell morphology, or the pattern and quantity of outgrowth. There was, however, one difference: Whereas extremely high doses of estrogen had no demonstrable effects, heavy doses of testosterone, 25 μg per ml or more, were injurious, either inhibiting initial outgrowth or severely damaging cells in an already established culture.

True growth, as indicated by the occurrence of mitosis, was slight in all cell cultures studied. No significant increase in growth occurred in response to hormone treatment. Survival in any given series of cell cultures, assessed at intervals for 1 month, was essentially equal in treated and untreated cultures, but not all series did equally well. In culturing many flank-organ tumors, we have acquired the tentative but distinct impression that tissue taken from young primary or transplanted tumors shows better growth than that taken from older sources. The reason for this is not clear, since when care is taken in the selection of explant tissue from larger, older tumors, *i.e.*, dissecting out small "active" nodules, the histological appearance of initial samples is very much the same as that of the smaller, younger tumor tissue.

## DISCUSSION

The reactivity of the flank-organ epithelioma to combined androgen and estrogen *in vitro*, although generally similar to that of the dependent renal carcinoma to estrogen (*5*), does differ significantly in one respect: Although neither of these tumors in dispersed-cell culture shows any

demonstrable response to medium-incorporated hormones—increased migratory activity, increased mitosis, altered patterns of outgrowth, or cellular characteristics in organotypic culture—the renal tumor manifests a strong dependency on estrogen, not only for growth but for survival beyond a limited time, whereas the flank-organ tumor survives fairly well without hormones, but cell division ceases unless the hormones are present.

Organotypic cultures of flank-organ tumor receiving 10 and 25 μg per ml of combined estrogen-androgen have more normal-looking surviving tissue, but appreciable quantities of quite normal tissue can be demonstrated in those that have received no hormones. It is only when mitosis is evaluated that an unmistakable effect of the hormones can be detected. Division figures are so rare in untreated cultures that one may, for all practical purposes, regard mitosis as having ceased. It is not improbable that the occasional figure seen may represent division of nontumor cell constituents, normal fibroblasts and macrophages. On the other hand, in hormone-treated cultures mitoses are relatively abundant during the first 10 days (text-fig. 1). It is true that mitosis falls rapidly after the 2d week, but it may be that this drop reflects other conditions of culture than hormone content. We find that the organ-culture method still serves best for short-term experimentation.

It is of interest and possible significance that the behavior of the flank-organ epithelioma in organ culture reflects to a measure its behavior *in vivo*. When exogenous hormones are withdrawn from animals bearing primary tumors or tumor transplants, tumor growth ceases, but decrease in size of tumor mass takes place only very slowly. Even after 12 months or longer, one can find well-developed tumor tissue. When hormones are reintroduced, growth starts anew. In this respect, the flank-organ tumor is different from the estrogen-dependent renal carcinoma (*5*) and other dependent hamster tumors under investigation (*1*): These involute quite rapidly after hormone withdrawal. Moreover, flank-organ tumors arise from peculiar laminated corpuscles in the tela subcutanea (hypodermis) underlying the organ. These microscopic corpuscles are normally present in old animals, but never grow beyond this size unless androgen is administered. Under the influence of androgen the laminated corpuscles grow to barely macroscopic size; further growth depends on the addition of estrogen. In organ culture, withdrawal of hormones produces a parallel, though short-term, response; growth ceases but the tissue remains otherwise relatively normal.

The reactivity of the dependent renal carcinoma (*2*), the flank-organ epithelioma, and other androgen-estrogen-induced, dependent hamster tumors [8] in organotypic culture suggests that "dependence" on hormones is a "local affair." The nature of this local affair and its relationships to the initial tumorigenic action of exogenous hormone(s) are unknown, and until more precise information is available, speculation is not warranted.

[8] An account of the behavior of an androgen-estrogen-induced, dependent leiomyoma of the ductus deferens in dispersed-cell and organotypic culture is in preparation.

That the hormone dependency of these hormone-induced hamster neoplasms seems to be reflected in organotypic culture is not surprising in view of the accumulated evidence that various isolated target tissues are capable of responding to hormone directly incorporated into the culture medium. Work of Gaillard on the effects of estrogen on accessory sex structures of immature mice (*11*), of Wolff (*12*), and Price and Pannabecher (*13*) on the differentiation of embryonic sex ducts in response to sex steroids and, more recently, work of Lasfargues and Murray (*14*) on embryonic mouse-mammary glands confronted with an array of hormones may be cited as examples which clearly demonstrate a "direct" relationship between responding tissue and hormone in an isolated system. Elias (*15*) and Elias and Rivera (*16*) present quite clear evidence that precancerous, hyperplastic nodules of mammary epithelium are sensitive to hormones in organ culture.

More enigmatic is the apparent inability of cells, from the hormone-dependent tumors with which we have been working, to respond in any significant fashion to hormones in dispersed cell culture. Reports concerning the effects of sex hormones, especially estrogen, are conflicting and difficult to interpret (*17*, *18*). The best agreement in the literature in this regard is to the effect that testosterone is apt to be inhibitory (toxic?) to many kinds of cells. Our failure to demonstrate an ability of cells from dependent tumors to react to hormones in cell culture has recently been paralleled by the findings of Franks and Barton (*19*). Not only did they fail to observe significant differences in behavior between hormone- and nonhormone-treated cell cultures of prostate of the mouse, but they showed the ultrastructure in both to be highly disorganized. They suggested that the loss of hormone sensitivity might be explained by this intracellular disorganization, or that it might be associated with disorganization of the tissue itself, *e.g.*, interruption of the epithelial-fibromuscular complex. It is interesting to note that some of the very first observations made *in vitro*, possibly predating Harrison's famous studies of amphibian embryonic nervous tissue, stressed the importance of stroma in influencing the behavior of epithelium in tissue culture (*20*). The importance of a physiological relationship between connective and epithelial tissues was stressed as early as 1865 (*21*).

## CONCLUSIONS

The androgen-estrogen-induced, dependent epithelioma of the flank organ of the Syrian hamster, which has no demonstrable sensitivity to hormones when grown in dispersed cell cultures, is dependent upon exogenous hormones in organotypic culture for growth and to a lesser extent for survival.

These observations are in agreement with those previously reported on the behavior of an estrogen-dependent carcinoma of the hamster kidney, with the exception that the latter failed to survive nearly as well without as it did with exogenous estrogen.

As suggested previously (*2*), the phenomenon of hormone dependency seems to be a "local affair" and not one which necessarily involves complex interrelationships in the whole animal. However, dependency does seem to require at least some degree of tissue organization: Growth and survival are similar in dispersed cell cultures regardless of the hormone content of the culture medium.

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# Effect of Azo-Dye Hepatocarcinogenesis on the Substrate Induction of Tryptophan Pyrrolase Activity [1,2]

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SUMMARY

**The substrate induction of tryptophan pyrrolase activity in the liver of rats was investigated to determine whether the ingestion of 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB) caused an early precancerous loss of this adaptive response. The results indicated that after 3, 4, or 5 weeks on diets containing 0.06 percent 3′-Me-DAB, the tryptophan pyrrolase activity after injection of 50 mg tryptophan per 100 g body weight was approximately sixfold greater than the activity in saline-injected animals on the same dietary regimen. On the other hand, the data indicated that the magnitude of the substrate-induced adaptive response among animals fed 3′-Me-DAB was usually less than that of animals on basal diets. Animals were fed 4-dimethylaminoazobenzene (DAB) or 4 derivatives of DAB with markedly different carcinogenic activities, and tryptophan pyrrolase activities were determined after substrate induction. Adaptive responses were observed with all 5 azo dyes, and the lowest adaptive response was fivefold higher than the saline-injected animals on the same dietary regimen. The lowest substrate induction was observed among animals fed a choline-deficient diet containing 0.25 percent ethionine, and supplementation of this diet with 0.6 percent methionine did not alter this response. Adaptation *in vitro* of tryptophan pyrrolase activity in a tissue-culture medium containing 4.16 mM tryptophan was consistently observed whether animals were maintained 4 weeks on a basal diet or this diet containing 0.06 percent 3′-Me-DAB or 4′-Me-DAB. From the results obtained, apparently there was no major alteration of the substrate induction of tryptophan pyrrolase activity during the early stages of hepatocarcinogenesis with azo dyes.—J. Nat. Cancer Inst. 27: 1503-1512, 1961.**

THE INFORMATION that has accumulated since the early description of the conversion of tryptophan to kynurenine (*1*) has shown that the enzyme was adaptive (*2*); that two different mechanisms operated to increase the enzyme activity, one stimulated by the substrate tryptophan, and one stimulated by several pituitary-adrenal hormones (*3*); and that these 2 mechanisms for adaptation operated independently (*4*). Recently

[1] Received for publication July 27, 1961.

[2] Throughout the paper 4-dimethylaminoazobenzene is designated DAB. The various C-monomethyl derivatives are designated 2′-Me, 3′-Me, or 4′-Me; the fluoro derivative is designated 4′-F.

[3] The excellent technical assistance of Boyd Howell and Otis Kile is gratefully acknowledged.

it was demonstrated that an activator, possibly iron porphyrin in the microsome fraction of liver, may be responsible for some of the increased tryptophan pyrrolase activity observed after tryptophan injections *in vivo* (*5*, *6*).

Investigations to determine whether tryptophan pyrrolase activity was present in malignant lesions of hepatic origin established that activity was missing in a number of hepatomas (*7–9*); however, measurable activity was apparently retained by some tumors of hepatic origin (*10–12*). The status of tryptophan pyrrolase activity during hepatocarcinogenesis with 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB) has received some attention. Fiala and Fiala (*13*, *14*) reported that tryptophan pyrrolase activity was not appreciably altered when animals were fed 3′-Me-DAB for 80 days. Their observation was subsequently confirmed (*9*), and evidence presented indicated that tryptophan pyrrolase activity was inducible in liver tissue with either tryptophan or histidine after a 90-day 3′-Me-DAB dietary regimen. On the other hand, Fiala and Fiala (*13*, *14*) observed that the adaptive response to tryptophan was essentially lost after 25 to 35 days on diets containing 3′-Me-DAB and concluded that 3′-Me-DAB either blocked (*13*) or inhibited (*14*) the synthesis of tryptophan peroxidase. From these observations, it appeared that 3′-Me-DAB blocked, in some manner, the induction of tryptophan pyrrolase by its substrate, but that this block was relieved in later stages of hepatocarcinogenesis. Since the block seemed transitory and since Civen and Knox (*4*) had established the independence of the hormone and tryptophan induction, it was of considerable interest to ascertain whether the hormone induction of tryptophan pyrrolase activity was operative under conditions in which the substrate induction had been eliminated by 3′-Me-DAB ingestion. In contradistinction to previous reports (*13*, *14*), preliminary experiments failed to indicate appreciable losses of adaptive response to tryptophan injection after 3, 4, or 5 weeks on diets containing 3′-Me-DAB. Thus it was of interest to determine (*a*) whether 3′-Me-DAB ingestion led to impairment of the substrate induction of tryptophan pyrrolase activity and (*b*) whether the apparent divergencies between these results and those previously reported (*13*, *14*) could be reconciled.

## MATERIALS AND METHODS

Female Holtzman rats 8 to 12 weeks old and weighing 150 to 200 g were used throughout the experimental work. They were maintained on Rockland rat chow, the semi-synthetic riboflavine-deficient diet described by Medes, Friedmann, and Weinhouse (*15*), or this diet with the choline concentration reduced to 1.5 g per kg. The derivatives of 4-dimethylaminoazobenzene (DAB) were synthesized from the appropriate precursors with the method of Giese, Miller, and Baumann (*16*), while DAB was purchased from Eastman Organic Chemicals, Rochester, New York. All azo dyes were added to the riboflavine-deficient diet at a concentration

of 0.06 percent, while the choline-deficient diet was supplemented with 0.25 percent ethionine,[4] 0.6 percent DL-methionine, or the combination of ethionine and methionine (*17*). In all experiments, animals were conditioned 7 days on the appropriate basal diet prior to the addition of DAB derivatives, ethionine, or ethionine plus methionine. For inductions with tryptophan, the dose was 50 mg per 100 g body weight (*4*) unless specified otherwise. The vehicle was 0.9 percent saline, and control animals received injections of the vehicle.

The assay method for tryptophan pyrrolase activity was essentially that of Knox (*18*), and the method was described in detail previously (*9*). Since in many of the experiments animals received parenteral L-tryptophan, substrate tryptophan was deleted from initial flasks and $1.25 \times 10^{-5}$ M sodium azide was added to inhibit the oxidation of endogenous tryptophan (*4*). In all assays, livers from 2 to 3 animals were pooled and homogenized. The tryptophan pyrrolase activity was expressed as μmoles kynurenine formed per g dry weight of liver per hour. Liver dry weights were determined by drying 1 ml aliquots of the liver homogenates at 110° C for 2 hours. Under these conditions, the liver dry weights of animals on basal diet usually exceeded that of animals ingesting 3′-Me-DAB. For instance, 1 ml aliquots of liver homogenates prepared from animals fed basal diet for 3 weeks yielded an average dry weight of 43.9 ± 0.6 mg (N = 46), while those from animals ingesting 3′-Me-DAB yielded an average dry weight of 38.7 ± 0.3 mg (N = 47).

Experiments, in which tryptophan induction *in vitro* was determined, were accomplished by the incubation of liver slices in the modified tissue-culture medium (*19*) described by Neuman and McCoy (*20*). For these experiments, the medium was prepared without tryptophan or human serum. L-Tryptophan was added to appropriate flasks at a concentration of 4.16 mM. The technique for the incubation of the liver slices was essentially that described by Civen and Knox (*4*). After incubation, the slices were homogenized and the tryptophan pyrrolase activity was determined in the usual manner.

## RESULTS

The first experiment (table 1) confirmed the preliminary observation that the ingestion of 3′-Me-DAB for periods of 3, 4, or 5 weeks did not eliminate the substrate-induced adaptive response of tryptophan pyrrolase activity. The magnitude of the adaptive response, however, was lower in the animals on diets containing 3′-Me-DAB. For instance, the tryptophan pyrrolase activity ratio (tryptophan-injected/saline-injected) ranged from 8.7 to 11.3 among the basal-diet animals throughout the 3- to 5-week feeding period, but among the animals ingesting 3′-Me-DAB, the activity ratio ranged from 6.1 to 6.5.

[4] DL-Ethionine was purchased from California Corporation for Biochemical Research, Los Angeles, Calif.

TABLE 1.—Effect of a 3-, 4-, or 5-week 3′-methyl-4-dimethylaminoazobenzene dietary regimen upon the substrate-induced adaptive response of tryptophan pyrrolase activity

| | Tryptophan pyrrolase activity* μmoles kynurenine/g dry weight/60 minutes | | |
|---|---|---|---|
| | Time diets were fed | | |
| | 3 Weeks | 4 Weeks | 5 Weeks |
| Basal diet | | | |
| Saline-injected | (1)† 3.53 ± — | (5) 2.84 ± 0.46‡ | (1) 2.90 ± — |
| Tryptophan-injected | (5) 31.23 ± 3.57 | (5) 31.62 ± 6.22 | (2) 25.09 ± 3.11 |
| Activity ratio tryptophan/saline | 8.9 | 11.3 | 8.7 |
| Basal + 3′-Me-DAB | | | |
| Saline-injected | (1) 3.55 ± — | (2) 2.89 ± 0.45 | (1) 3.62 ± — |
| Tryptophan-injected | (6) 22.90 ± 4.76 | (2) 18.93 ± 4.79 | (2) 22.11 ± 3.88 |
| Activity ratio tryptophan/saline | 6.4 | 6.5 | 6.1 |

*Conditions for enzyme assay: 0.3 ml, 0.03 M tryptophan; 1 ml, 0.1 M phosphate buffer; 0.1 ml, 0.1 M glucose; 1 ml homogenate; 1.6 ml water. Activities were calculated on OD final−OD initial = ΔOD. In all initial reaction mixtures, tryptophan was deleted and 0.3 ml $1.25 \times 10^{-4}$ M sodium azide was added.

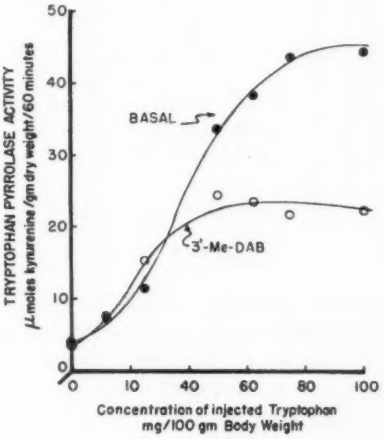
Conditions for animals: 3′-Me-DAB in diets at 0.06 percent; tryptophan injected at 50 mg per 100 g body weight. Animals were killed 5 hours after injection.

†Number of experiments; for each experiment 2 to 3 livers were pooled.

‡Standard deviation.

Since a definite difference in the magnitude of the adaptive response apparently existed between the two dietary groups, it was possible that under other conditions of induction these differences might be more pronounced and might lead to the conclusion that the adaptive response was deleted in the early stages of hepatocarcinogenesis with 3′-Me-DAB (*13*, *14*). Lee (*21*) showed that a linear relationship existed between the amount of tryptophan injected and the maximum level of tryptophan pyrrolase activity achieved over a concentration range of 10 to 100 mg per 100 g body weight. In view of this relationship, it seemed appropriate to ascertain the effect of the concentration of injected tryptophan upon the liver pyrrolase activity of animals maintained on a basal diet or this diet containing 0.06 percent 3′-Me-DAB. The data are shown in text-figure 1. When the concentration of injected tryptophan was less than 50 mg per 100 g body weight, the livers of both basal- and 3′-Me-DAB-fed animals showed essentially identical adaptive responses. When concentrations of 50 mg per 100 g body weight or greater were injected, the basal-fed animals consistently showed greater adaptive responses than did the 3′-Me-DAB-fed animals. On the other hand, although the magnitude of the adaptive response was lower in the 3′-Me-DAB-fed animals, adaptation was consistently observed. Fiala and Fiala (*13*, *14*) injected 78 mg of tryptophan into each animal. If a body weight of 150 to 200 g were assumed such a dosage would approximate 50 mg per 100 g body weight. In this laboratory it has been consistently observed that basal-

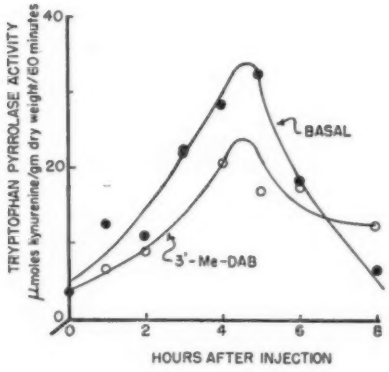
diet animals maintain a higher weight average than do animals fed 3′-Me-DAB. Dyer and Morris (*22*) noted a similar weight differential in animals fed *N*-2-fluorenylacetamide. In view of these considerations, the carcinogen-fed animals in the experiments reported by Fiala and Fiala (*13*, *14*) probably received tryptophan injections equaling or exceeding 50 mg per 100 g body weight. Since the dose-response curves shown in text-figure 1 indicate significant adaptation of the tryptophan pyrrolase activity throughout a wide range of tryptophan concentrations in both basal- and 3′-Me-DAB-fed animals, it would seem that the failure to observe adaptation in 3′-Me-DAB-fed animals (*13*, *14*) was not associated with inadequate concentrations of injected tryptophan.




TEXT-FIGURE 1.—Comparison of the effect of injected tryptophan concentration on the adaptive response of the tryptophan pyrrolase activity in livers from animals fed basal diet or basal diet containing 3′-Me-DAB. With the exception of the lowest concentration (12.5 mg/100 g body weight), each point represents the averages of 3 to 5 experiments, but the lowest concentration represents the average of 2 experiments. For each experiment, livers from 3 animals were pooled.

The time course of the substrate induction of tryptophan pyrrolase activity was studied by several groups (*4*, *21*, *23*, *24*) with the general agreement that maximum induction occurred at 4 to 6 hours after tryptophan injections. Civen and Knox (*4*) established that the concentration of free tryptophan in the blood and liver reached maximal values approximately 30 minutes after injection and was essentially normal at the time maximum induction of tryptophan pyrrolase activity was observed. Feigelson, Dashman, and Margolis (*23*) reported that the decay of induced enzyme activity in rat liver followed the kinetics of a first-order reaction with a calculated T ½ of 2.4 after tryptophan induction. In view of these findings, it seemed appropriate to determine whether the time course of induction in animals fed 3′-Me-DAB compared favorably with that of animals maintained on basal diets. The effect of the time interval between tryptophan injection and determination of the tryptophan pyrrolase activity is shown in text-figure 2. Animals maintained on either dietary regimen for 3 weeks received injections of 50 mg tryptophan per 100 g body weight and were killed at hourly intervals through 6 hours and at 8 hours. Both groups showed optimum induction at 4 to 5 hours. Although animals fed 3′-Me-DAB showed a response having less magnitude

compared to animals maintained on basal diets, the maximum response among the 3′-Me-DAB-fed animals was increased approximately fivefold over animals given injections of saline. Since in the work of Fiala and Fiala (*13*, *14*), animals were killed 6 hours after tryptophan injection, the time-course data shown in text-figure 2 would largely discount this factor as a cause for a failure to observe substrate induction in 3′-Me-DAB-fed animals.




TEXT-FIGURE 2.—Comparison of the effect of time after injection on the adaptive response of the tryptophan pyrrolase activity in livers from animals fed basal diet or basal diet containing 3′-Me-DAB. From 2 hours through 6 hours each point represents the average of 4 experiments, while the 1 and 8 hour points represent the average of 2 experiments. For each experiment, livers from 3 animals were pooled.

Since the data consistently indicated that the livers of animals fed 3′-Me-DAB displayed adaptive responses, it was of interest to ascertain whether other DAB derivatives or ethionine would eliminate the adaptive response to tryptophan. These data are shown in table 2. Animals were maintained 4 weeks on diets containing the various C-monomethyl derivatives of DAB and received injections of 50 mg tryptophan per 100 g body weight. The azo-dye-fed animals showed adaptive responses ranging from fivefold to nearly 13-fold increases in tryptophan pyrrolase activity compared to the controls given saline injections. Animals fed DAB, 3′-Me-DAB, or 4′-F-DAB had an adaptive response that was of a lesser magnitude than that of the basal group or the groups fed 4′-Me-DAB or 2′-Me-DAB. However, despite the lesser magnitude, there appeared to be no question concerning the presence of an adaptive response in the precancerous livers of animals fed carcinogenic azo dyes. When animals were fed ethionine or ethionine plus methionine at concentrations reported to prevent ethionine-induced carcinoma of the liver (*17*), the adaptive responses were markedly lower. Nevertheless, the tryptophan pyrrolase activity of the animals receiving tryptophan injections was significantly higher (ethionine $P<0.01$, ethionine plus methionine $P<0.02$) than the activity in control animals given saline injections and maintained under the same dietary conditions.

Since Civen and Knox (*4*) demonstrated substrate induction *in vitro* of tryptophan pyrrolase activity, it was of interest to determine whether the previous dietary status of the rat would alter the induction *in vitro*. Liver slices were prepared from animals maintained 4 weeks on Rockland

TABLE 2.—Effect of a spectrum of liver carcinogens on adaptive response of tryptophan pyrrolase activity

| 4-Week dietary regimen† | Tryptophan pyrrolase activity* μmoles kynurenine/g protein/60 minutes | | |
|---|---|---|---|
| | Saline-injected | Tryptophan-injected‡ | Ratio tryptophan/saline |
| Basal | (7)§ 2.95 ± 0.33‖ | (8) 28.83 ± 3.99 | 9.8 |
| Basal plus | | | |
| 4′-Me-DAB | (3) 2.60 ± 0.14 | (3) 28.20 ± 6.06 | 10.8 |
| 2′-Me-DAB | (3) 1.42 ± 0.10 | (3) 17.71 ± 3.19 | 12.6 |
| DAB | (3) 2.24 ± 0.21 | (3) 14.59 ± 0.47 | 6.5 |
| 3′-Me-DAB | (4) 3.24 ± 0.86 | (5) 19.66 ± 2.24 | 6.1 |
| 4′-F-DAB | (3) 1.99 ± 0.33 | (3) 10.16 ± 0.97 | 5.1 |
| Basal, choline-deficient | (3) 2.76 ± 0.68 | (3) 37.00 ± 7.15 | 13.4 |
| Basal, choline-deficient plus | | | |
| Methionine | (3) 3.31 ± 0.71 | (3) 32.05 ± 4.18 | 9.7 |
| Ethionine | (3) 3.56 ± 0.47 | (3) 8.45 ± 0.83 | 2.5 |
| Ethionine + methionine | (3) 3.20 ± 0.55 | (3) 5.33 ± 0.10 | 1.7 |

*Conditions: *See* table 1.
†The derivatives of DAB were fed at 0.06, methionine at 0.6, and ethionine at 0.25 percent.
‡Tryptophan injected at 50 mg per 100 g body weight; animals killed at 5 hours.
§ Number of experiments; for each, 2 to 3 livers were pooled.
‖ Number standard deviation.

chow, basal diet, basal diet with 0.06 percent 4′-Me-DAB, or basal diet with 0.06 percent 3′-Me-DAB. The data from these experiments are shown in table 3. Regardless of previous dietary experience, the tryptophan pyrrolase activity after incubation in the presence of tryptophan was higher than that of the nonincubated slices. With but one exception the means were significantly different at a probability level exceeding 0.05 percent. The exception, animals fed 3′-Me-DAB, was significantly different at a probability level exceeding 0.10 percent. Thus, these data further supported the contention that the mechanisms responsible for demonstration of tryptophan pyrrolase activity were not deleted during the early stages of 3′-Me-DAB carcinogenesis.

## DISCUSSION

The data in the present paper consistently support the conclusion that 3′-Me-DAB carcinogenesis caused no deletion of the adaptive response of tryptophan pyrrolase activity during the first 3 to 5 weeks. On the basis of the data in this report and in a previous report (*9*) it would appear unlikely that any major alterations of the enzymic conversion of tryptophan to kynurenine occur in azo-dye-induced precancerous livers.

Reasons for the discrepancies between the present data and those previously reported (*13, 14*) must remain somewhat conjectural. From the data reported (*13*), some of the discrepancy may be associated with the relatively low adaptive response that was consistently observed. On

TABLE 3.—Effect of azo-dye ingestion on induction of tryptophan pyrrolase activity in liver slices incubated in a tissue-culture medium

| Dietary regimen† | Tryptophan pyrrolase activity* μmoles kynurenine/g dry weight/60 minutes | | | |
|---|---|---|---|---|
| | I<br>No incubation | II<br>Incubation in absence of tryptophan | III<br>Incubation with 4.16 mM tryptophan | Student's *t* comparisons |
| Laboratory ration | (7)‡ 4.36 ± 0.48§ | (7) 5.32 ± 1.03 | (7) 9.48 ± 1.78 | I vs III $P<0.05$<br>II vs III $P<0.10$ |
| Basal diet | (9) 3.16 ± 0.24 | (8) 2.87 ± 0.42 | (9) 4.92 ± 0.39 | I vs III $P<0.01$<br>II vs III $P<0.01$ |
| Basal + 4′-Me-DAB | (8) 1.90 ± 0.18 | (7) 2.25 ± 0.38 | (8) 4.66 ± 0.55 | I vs III $P<0.01$<br>II vs III $P<0.01$ |
| Basal + 3′-Me-DAB | (10) 3.99 ± 0.26 | (10) 2.30 ± 0.47 | (10) 5.29 ± 0.43 | I vs III $P<0.10$<br>II vs III $P<0.01$ |

*Conditions for the assay of tryptophan pyrrolase activity, *see* table 1. For liver slice incubation conditions *see* text.
†The azo dyes were added at a concentration of 0.06 percent. Diets were fed 4 weeks.
‡Number of experiments. For each experiment liver slices from 3 animals were pooled.
§ Mean ± standard deviation.

the basis of the specific activities reported (*13*), the adaptive response among the control animals rarely exceeded a twofold increase. When we considered uncorrected activities based on the amount of kynurenine formed with 1 ml of liver homogenate (*13*), the differential between untreated animals and those receiving injections of tryptophan rarely exceeded threefold. Since in the present work, the adaptive response in animals fed 3′-Me-DAB was usually less than that observed in animals fed basal diet, a low induction in control animals might make detection of adaptation among the 3′-Me-DAB-fed animals difficult.

The data in previous reports (*9*, *13*, *14*) established that tryptophan pyrrolase activity did not undergo appreciable change during hepatocarcinogenesis with 3′-Me-DAB, but this enzyme activity was not demonstrable in either the primary or the transplanted lesions that subsequently developed (*9*). The present study established that the ability to induce tryptophan pyrrolase activity with its substrate was not lost during the early stages of hepatocarcinogenesis with 3′-Me-DAB. In a previous report (*9*), induction was readily demonstrated after 90 days on 3′-Me-DAB and in the liver tissue adjacent to primary lesions. Attempts to induce tryptophan pyrrolase activity in 3′-Me-DAB transplanted lesions were unsuccessful, and whether inductions were fruitful in 3′-Me-DAB primary lesions would appear questionable (*9*). From these considerations it seems that the only detectable alteration in the tryptophan pyrrolase activity of liver that preceded the development of overt malignancies after 3′-Me-DAB ingestion was a slight decrease in the magnitude of the substrate-induced adaptation. Cantero (*25*) held that guideposts of metabolic changes in precancerous liver could be recognized on a tissue level as well as at the cellular level. Thus, if tryptophan pyrrolase were subjected to preneoplastic change, apparently these changes occurred primarily at the cellular rather than at the tissue level.

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# Announcement

## Manuscripts Approved for JNCI, October 15 to November 15, 1961

"Transplantation studies on estrogen-induced interstitial-cell tumors of the testis in mice," by G. Klein and K. E. Hellström.

"Comparative chromosomal study of 31 cultured mammalian cell lines," by J. J. Clausen and J. T. Syverton.

"Testicular teratomas in fetal mice," by Leroy C. Stevens.

"A comparative study of the kinetics of cellular proliferation of normal and tumor tissues with tritiated thymidine. I. The dilution of the label and the migration of labeled cells," by Renato Baserga and W. E. Kisieleski.

"Inhibition of growth of amethopterin-sensitive and amethopterin-resistant pairs of lymphocytic neoplasms by dietary folic-acid deficiency in mice," by Michael Potter and George Briggs.

"Polyoma virus dose-response studies in mice. I. Dwarfing, tumor incidence, and antibody response of animals infected in the neonatal period," by Paul M. Kraemer.

"Polyoma virus dose-response studies in mice. II. Characteristics of induced salivary-gland tumors," by Paul M. Kraemer.

"Studies on the exchange of fluids between host and tumor. III. Regulation of blood flow in hepatomas and other rat tumors," by Pietro M. Gullino and Flora H. Grantham.

"Transplantable human tumors in experimental chemotherapy: Effects of actinomycins on H.S. #1 and H.Fp. #3 in the rat," by M. N. Teller, P. C. Merker, J. E. Palm, and George and G. W. Woolley.

"Quantitative studies on the induction of tolerance of skin homografts and on runt disease in neonatal rats," by R. E. Billingham, V. Defendi, W. K. Silvers, and D. Steinmuller.

"Further studies on antibody status of mice and response of their litters to parotid tumor (polyoma) virus," by L. W. Law, W. P. Rowe, Janet W. Hartley, and C. J. Dawe.

"Induction of carcinoma and papilloma of the tracheobronchial mucosa of the Syrian hamster by intratracheal instillation of benzo[*a*]pyrene," by Katherine M. Herrold and Lucia J. Dunham.

"A cytogenic study of embryonic chick pancreas. I. The exocrine tissue," by Robert F. Zeigel.

"Effect of injection of foreign bone marrow on the lymphatic tissue of normal mice," by C. C. Congdon.

"Chromosome observations on three epithelial-cell cultures derived from carcinomas of the human cervix," by Nelly Auersperg and Annette P. Hawryluck.

# ERRATUM

*NCI Monograph No. 6, pp. 220–221.*

The text-figure titles for text-figures 64 and 65 should be reversed.

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